UNIVERSITY OF CALIFORNIA SAN DIEGO

The Role of DNA Methylation in Driving the Microglial Inflammatory Response

A thesis submitted in partial satisfaction of the requirements for the Master of Science

in

Bioengineering

by

Keya Trivedi

Committee in charge:

Professor Paula Desplats, Chair
Professor Sheng Zhong, Co-Chair
Professor Adam Engler

2023

The thesis of Keya Trivedi is approved, and it is acceptable in quality and form of publication on microfilm and electronically.

University of California San Diego

2023

Table of Contents

Thesis Approval Page. ..... *iii*
Acknowledgments ..... *vi*
Abstract ..... *vii*
Introduction *1*
Molecular Pathology of AD *2*
Amyloid 2
Tau 4
Neuroinflammation in AD *5*
Microglia *6*
Epigenetics in AD *8*
Previous work in Desplats lab *10*
Methods *12*
Cell culture and treatment 12
Amyloid-β Peptide and LPS treatments 12
Real-time PCR 15
Measuring 5mC and 5hmC levels using ELISA 15
C35 Treatment 15
Results *16*
Characterization of the response to LPS and Aβ 1-42 fragments in HMC3 human primary immortalized microglia in culture 16
Microglial activation is associated with global changes in DNA methylation 22
Evaluation of Global Methylation changes associated with Inflammation in HMC3 25
Effects of TET Inhibitor C35 on Basal Methylation Levels in HMC3 Cells 26
TET Inhibitors 27
Effects of C35 treatment on methylation in HMC3 cells in response to LPS 29
Effects of 24-hour C35 treatment on methylation in HMC3 cells in response to Aβ 30
Effects of C35 on the inflammatory response to LPS and Aβ in HMC3 cells 31
Conclusion *33*
Future Directions *34*
Time and Dose Response curves for C35 34
Determination of DNMTs and TETs activities 34
Triculture experiments with microglia, astrocytes, and neurons 34
In vivo experiments with AD-mice 34

Sequencing for gene-specific methylation 34
Limitations of the study *35*
Differences in microglial behavior in vitro vs. in vivo 35
References *36*

Acknowledgments

First and foremost, I am deeply thankful to Dr. Desplats for her invaluable mentorship throughout this journey. Her guidance, expertise, and support have been pivotal in shaping my research. I would also like to thank the members of the Desplats Lab for their helpful input, collaboration, and camaraderie. I am also grateful to Dr. Sheng Zhong and Dr. Adam Engler for their time and constructive feedback. I would also like to thank Dr. Charles Richard John Lalith for his expertise, and training throughout this process. Lastly, I would like to thank my family and friends for their endless love, encouragement, and belief.

ABSTRACT OF THE THESIS

The Role of DNA Methylation in Driving the Microglial Inflammatory Response

by

Keya Trivedi

Master of Science in Bioengineering

University of California San Diego, 2023

Professor Paula Desplats, Chair
Professor Sheng Zhong, Co-Chair

Alzheimer's Disease (AD) is a severe neurodegenerative disease characterized by cognitive dysfunction caused by synaptic loss. Its pathogenesis is driven by the development of extracellular plaques due to the accumulation of misfolded amyloid-beta (Aβ) protein and the formation of neurofibrillary tangles (NFTs) caused by the aggregation of misfolded Tau. Due to protein aggregation and cell loss during neurodegeneration, microglia, the innate immune cells of the central nervous system (CNS), acquire a unique activation phenotype that triggers inflammation. My thesis aims to explore the role of DNA methylation in driving these inflammatory responses using human-immortalized microglial cells. We

profiled these cells for various inflammatory and disease-associated microglia (DAM) markers following *in-vitro* exposure to either lipopolysaccharide to model bacterial infections or to amyloid-beta to model neuroinflammation in AD. To investigate global methylation mediating these inflammatory changes, we measured 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) levels at specific time points. We also explored the potential of TET inhibitors as a therapeutic approach to reduce neuroinflammation by measuring the changes in 5-mC and 5-hmC levels as well as inflammatory markers in cells treated with the TET-inhibitor C35. Taken in all, my results suggest that DNA methylation plays an essential role in eliciting the inflammatory response of microglia to different stimuli, thus implicating this epigenetic mechanism in the pathology of AD.

Introduction

Neurodegenerative disorders such as Alzheimer's disease (AD), Frontotemporal dementia (FTD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and others are among the top 10 leading causes of death globally [13]. As of a 2021 report, it is estimated that the number of Alzheimer's cases worldwide is as high as 24 million. Older people, especially those over the age of 65, are prone to develop AD, although it can occur in younger populations as well [36]. AD is the most common type of dementia, a condition widely characterized by a progressive decline in memory, cognition, language, and hand-eye coordination, as well as behavioral changes in personality and ability to do basic tasks. The clinical presentation of AD shows a high degree of neuronal loss in the brain, specifically in the hippocampus and the cerebral cortex [39]. In addition to neuronal loss, two pathological hallmarks drive AD: the accumulation of amyloid-β (Aβ) protein that forms extracellular plaques and the formation of neurofibrillary tangles (NFTs) caused by the aggregation of Tau protein inside neurons [35] (Figure 1). An accumulation of misfolded Aβ peptide causes amyloid plaques, and the Tau protein is a microtubule-associate protein found in neurons of the CNS that becomes prone to aggregation when it is misfolded because of hyperphosphorylation [29].

There is currently no known cure for AD, although some treatments can temporarily alleviate some of its mental and physical symptoms. Memory loss is the major symptom of AD. However, motor functions and daily activities are also compromised, and conditions such as social withdrawal and depression are elevated [25]. AD not only impacts the patient's life but also those around them because of the significant burden on them as caregivers, along with the rising cost of treatment and medical facilities. As the population grows older, the risk of being affected by AD also rises, creating a critical need to improve our understanding of what causes neurodegenerative diseases and develop new approaches for treatment and prevention.

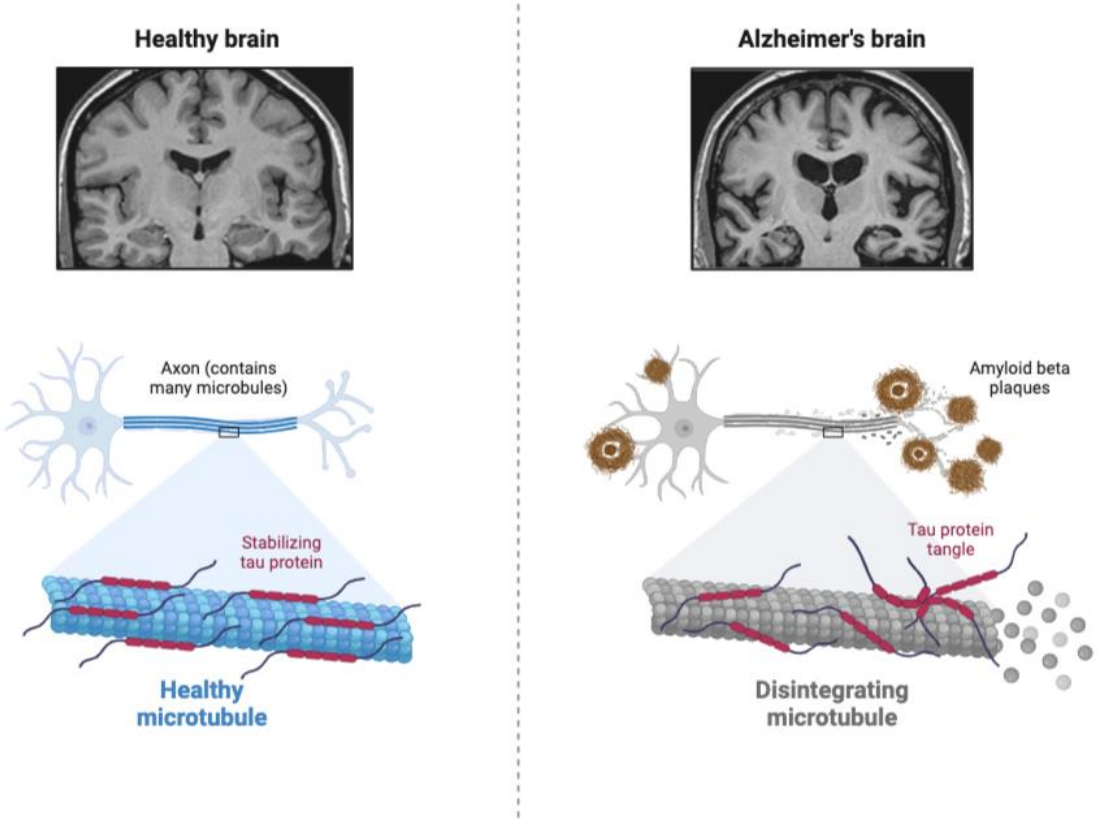


Figure 1: Alzheimer's disease pathogenesis, characterized by Amyloid plaques and neurofibrillary tangles

Source: Created by Biorender.com

Molecular Pathology of AD

Amyloid

AD has two main neuropathological features: amyloid plaques and neurofibrillary tangles (NFTs). In addition, other changes, such as cerebral atrophy, neuronal loss, and inflammation, are also observed [40]. Genome-wide associated studies (GWAS) have identified Aβ peptides as linked to the onset of familial AD, making them a central research focus [46]. Different forms of Aβ have been identified over the years, such as fibrillar Aβ, insoluble protofibrils, and soluble oligomers, each having varying neurotoxicity effects [17]. Accumulating evidence from the past few decades suggest that Aβ accumulation in neurons contributes majorly to AD pathogenesis, and thus many experimental therapies focus on reducing Aβ

peptide levels in the brain. However, the complete molecular mechanism of Aβ neurotoxicity remains unclear [4].

Amyloid protein was first purified and characterized from the vasculature in the brain of AD patients. The amyloid precursor protein (APP) was subsequently cloned in 1984 at the University of California San Diego [25]. APP is a type I transmembrane protein that is proteolytically processed by the α-, β-, and γ-secretases, resulting in the disease-associated isoform Aβ 1-42, which constitutes the senile plaques formed during AD and accounts for 5–10% of the total amount of Aβ that is produced [25, 34] (Figure 2). Moreover, mutations in genes encoding for APP and presenilin (PSEN1 and PSEN2), linked to familial early-onset AD, have been found to enhance the production of Aβ peptides and promote their aggregation. These genetic findings have led to Aβ peptide becoming a central area of focus in AD research [18, 22].

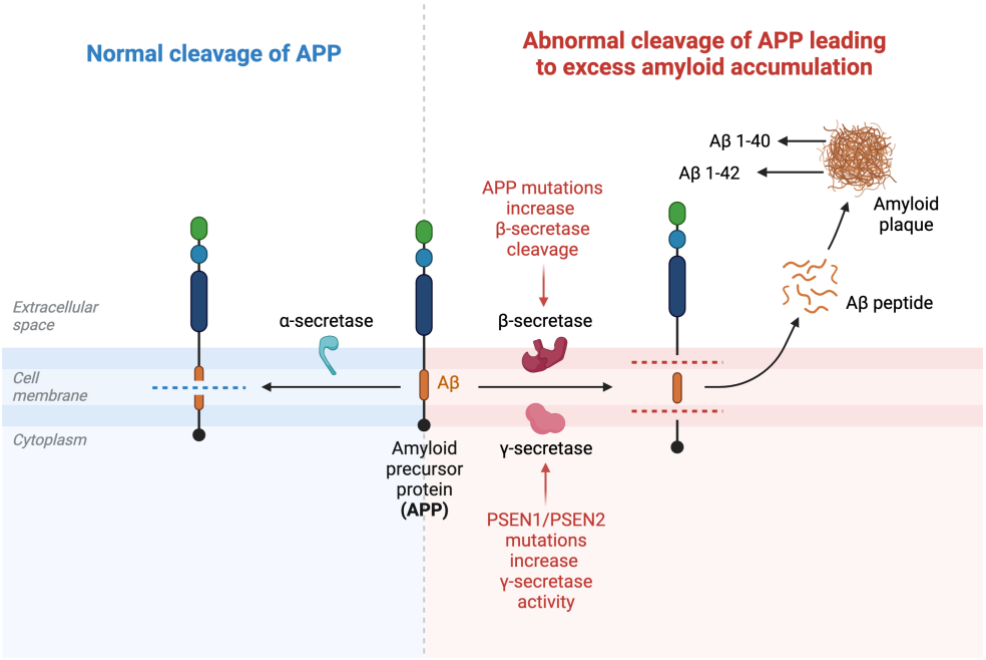


Figure 2: The APP protein and its cleavage by secretases, forming proteolytic fragments of Aβ 1-40 and Aβ 1-42

Source: Created with BioRender.com

The mechanism by which AD progresses has been at the center of research for many years. Initially, it was believed that Aβ was produced at the cell's outer membrane, where it formed aggregates of insoluble β-

pleated amyloid fibrils and then released into the extracellular environment, leading to neurotoxic effects [42, 53]. More recently, advances in electron and atomic microscopy have shown that the intermediate products of Aβ fibril formation, such as lower molecular weight Aβ oligomers and high molecular weight protofibrils that form stable structures, have a more significant neurotoxic effect and are more harmful to synaptic plasticity than Aβ fibrils [47, 57]. Overall, it is accepted that three major groups exist among the various Aβ isoforms- monomers, soluble oligomers, and insoluble fibrils. In addition, it is considered that soluble oligomers correlate better with cognitive dysfunction in the AD brain [45] and that their toxicity is dependent on size and diffusion, with smaller units having greater bioreactivity and hence greater detrimental effects [47]. These soluble oligomers facilitate their effects by interacting with cell surface receptors, causing membrane disruption and ion dysregulation [47]. They have also been shown to inhibit long-term potentiation (LTP) and are associated with weakening and loss of synapses as well as oxidative damage [9], leading to an increase in cognitive impairment in AD patients [18].

Tau

Research on the role of Aβ in AD has advanced faster than research on tau. Identifying genetic mutations responsible for autosomal familial AD led to the formulation of the 'amyloid cascade hypothesis,' which shifted the research focus towards Aβ [18]. Research exploring the biochemistry of APP and presenilins has greatly improved our understanding of molecular mechanisms involved in Aβ production and their physiological effects [39]. These developments have subsequently led to more focus on the development of therapies targeting the Aβ pathway aimed at slowing disease progression. On the contrary, tau has received comparatively less attention as a therapeutic target, indicated by fewer clinical trials surrounding it [39]. However, in recent years, AD research focused on tau has gained significance, with more studies using mice models exhibiting tau pathology [60].

Tau is encoded by the MAPT gene and alternate splicing of that results in six different tau isomers [30]. It is a microtubule-associated protein found in axons and in a healthy brain, it is highly soluble and exists in

an unfolded state which allows it to interact with tubulin and regulate its promotion and stabilization [35]. This interaction of tau with microtubules is regulated by post-translational modifications such as phosphorylation, ubiquitination, oxidation, etc., out of which phosphorylation is the most studied. What makes tau an exciting focus of research is that besides its involvement in AD, it also plays a vital role in several other CNS disorders such as Frontotemporal dementia (FTD), Pick's disease (PID), and corticobasal degeneration (CBD), together known as tauopathies [3].

Tau undergoes phosphorylation on a few sites in a normal brain [16], but during AD, it becomes hyperphosphorylated due to an imbalance in the activity of tau kinases such as GSK3 [3]. This results in a reduced affinity of tau for microtubules and increased resistance to degradation and removal via ubiquitination [1]. Consequently, this leads to an accumulation of hyperphosphorylated tau in neurons which aggregate into neurofibrillary tangles (NFTs) [35].

However, in this study, we have focused on the effects of Amyloid-beta in triggering microglial activation.

Neuroinflammation in AD

Besides the two pathological hallmarks of AD discussed above, there is ample evidence that AD pathogenesis is not just restricted to neurons but has substantial implications on immunological mechanisms as well. Misfolded and aggregated proteins bind to pattern recognition receptors (PRRs) on microglia, triggering an immune response characterized by the release of inflammatory cytokines, which affect neuronal function and cause cell death, thus contributing to disease progression and severity [20, 32]. Many recent studies have shown that genes such as TREM2 and CD33, which regulate immune responses, are associated with a high risk of developing AD, highlighting the significance of studying neuroinflammation as a major hallmark of AD [20].

Microglia

Neuroinflammation in the brain is mediated by microglia, the resident immune cells of the CNS. In a healthy brain, microglia clear pathogens and cellular debris and also provide ions, metabolites, trophic factors, etc., that support tissue maintenance [32]. They also contribute to the protection and remodeling of synapses for proper maintenance and plasticity of neuronal circuits [23]. Injury or pathological triggers such as neuronal death and protein aggregates cause microglia to extend their processes to the injury site and initiate an immune response. Microglia also remove unwanted neurons and synapses, thus helping in modeling neural circuits [46]. In case of pathogenic invasion, they become activated and release pro-inflammatory cytokines (Figure 3), including tumor necrosis factor-alpha (TNF-α), and interleukins 6 and 1β (IL-6 and IL-1β), which further induce the release of various secondary inflammatory signals [46].

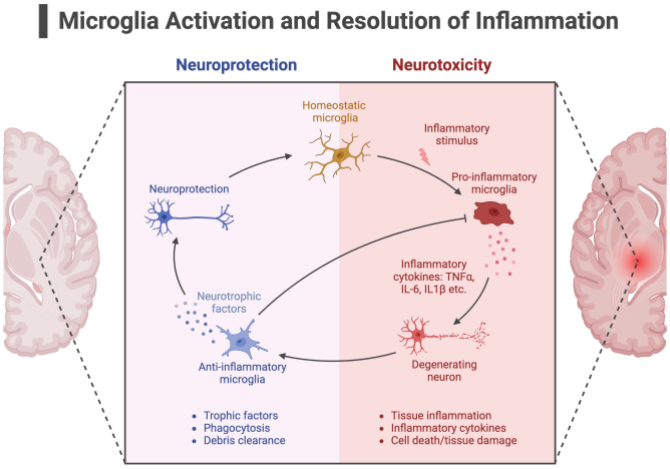


Figure 3: Microglia activation and resolution of inflammation in the human brain

Source: Created with BioRender.com

Microglia in AD

Knowing how microglia function in a healthy brain, we shall now look at how microglia react in case of a pathological trigger such as Amyloid-β (Aβ). Microglia help to remove Aβ by two main mechanisms- either

by efflux of the soluble oligomers or via proteolytic cleavage of the soluble oligomers and insoluble fibrils [29]. Recently, comprehensive transcriptomic analyses, such as single-cell RNA sequencing (sc-RNA-seq) of microglia in neurodegenerative conditions, have defined Disease-associated microglia (DAM), a subset of microglia showing a unique transcriptional and functional signature [12, 58] (Figure 4). Importantly, DAM expresses many genes which were found in genome-wide association studies (GWAS) as linked to AD and other neurodegenerative conditions, such as Apolipoprotein E (APOE), triggering receptor expressed on myeloid cells (TREM2), and cluster of differentiation (CD33) among others [12, 58]. DAM are characterized by a reduced expression of microglial homeostatic genes such as *P2RY12, CD33, CX3CR1, and TMEM119* (Figure 4) and an upregulation of genes involved in phagocytosis and lipid metabolism, including AD risk genes such as *CTSD, LPL, and TREM2* among others [6, 12, 58] (Figure 4). In addition, DAM differentiation has been shown to be a two-step process- Stage 1 DAM consists of genes that are activated independent of TREM2, and Stage 2 DAM consists of genes that are dependent on the TREM2 signaling cascade (Figure 4) [6].

However, whether this DAM phenotype is beneficial or detrimental to neurodegeneration is still a topic of debate. Research suggests that activated microglia help to clear Aβ buildup in an AD brain by forming a protective barrier around the plaques, converting the toxic soluble Aβ oligomers into insoluble fibrils and blocking them from traveling to nearby cells [10]. However, there is also contrasting evidence showing that the localization of fewer microglia caused by a deficiency in the C3 complement cascade regulating innate immunity alleviated the levels of pro-inflammatory cytokines and improved memory and learning in AD mice models [50]. Further, it is still unclear whether DAM respond differently in early versus late stages of AD [58]. Thus, studying DAM mechanisms, associated signaling cascades, and their role in disease pathogenesis is critical for neurodegeneration research.

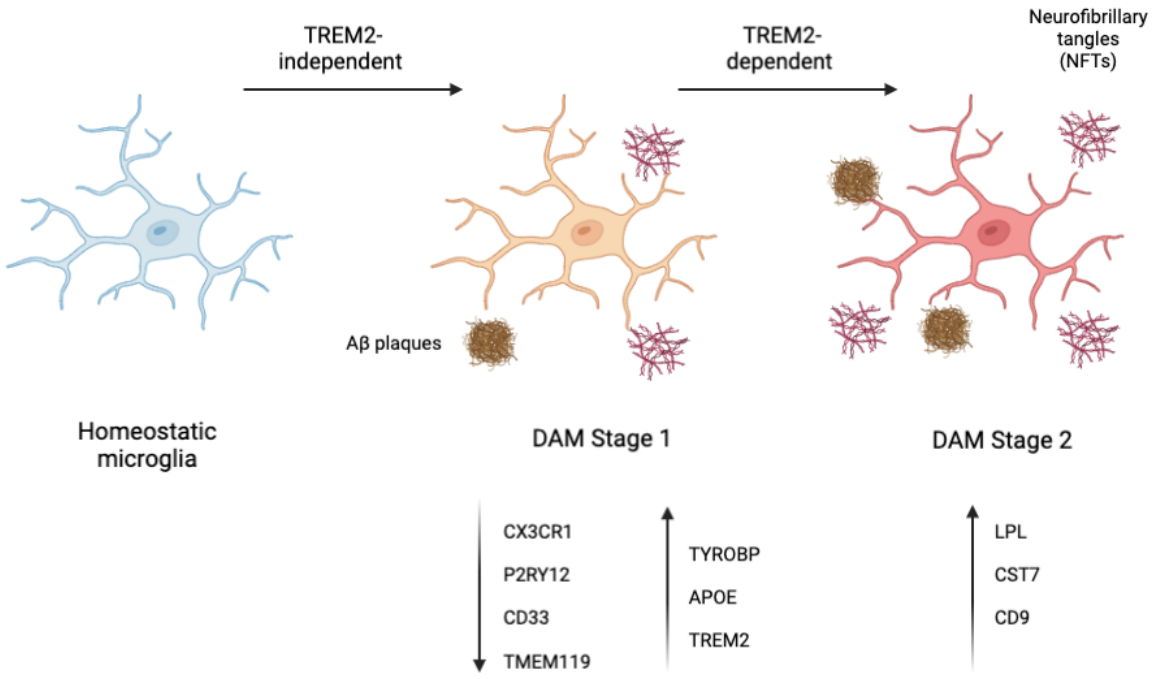


Figure 4: A two-step model of DAM induction showing downregulation of homeostatic microglial genes and upregulation of phagocytosis, lipidosis, and AD-risk genes

Source: Created with BioRender.com

Epigenetics in AD

Epigenetic mechanisms such as post-translational histone modifications, chromatin remodeling, DNA methylation, and non-coding RNA expression are reversible modifications that can regulate gene expression without changing an organism's actual DNA sequence [13]. Several studies have demonstrated the relationship between epigenetic dysregulations and AD [49]. However, their exact contribution to disease progression and their role in microglial activation remains relatively unknown.

DNA methylation is a dynamic, cyclic process that involves the addition of a methyl group to the C5 position of a cytosine ring in a CpG dinucleotide, by the DNA-methyl transferase (DNMT) enzyme family, resulting in 5-methyl-cytosine, or 5mC [5, 24] (Figure 5). DNMT1 regulates the maintenance of methylation patterns by ensuring that the methylome of the parent strand is correctly copied to the new

daughter strand [40]. The other two functional DNMTs- DNMT3a and DNMT 3b carry out what is known as *de novo* methylation, which is the process of adding a new methyl group to previously unmodified cytosine [40]. Several studies have established the connection between changes in 5mC levels and DNA stability and gene expression [44]. There is also evidence that 5mC plays a role in aging, given that global 5mC levels have been shown to increase during the process [11]. Although DNA methylation is regarded as a stable feature, 5-methylcytosine (5mC) can be converted to 5-hydroxymethylcytosine (5hmC) by the Ten-Eleven Translocation (TET) family of enzymes, which share highly similar catalytic domains that bind DNA [44]. Structurally, the three TET enzymes are similar and share a conserved C-terminal catalytic domain containing the metal ion ligand-binding residues required for the 5mC to 5hmC oxidation reaction and a less conserved N-terminal region [2, 28].

5hmC is an intermediate stage of DNA de-methylation and is associated with an increased gene expression as opposed to 5mC, which suppresses gene expression [11]. 5hmC has been shown to be involved in important physiological processes such as cell differentiation and neural development [52] and has also been shown to increase during the process of aging [8]. Thus, over the last few years, evaluating 5hmC levels in the context of neurodegenerative disorders has gained significant traction [11]. However, the exact implication of 5mC and 5hmC levels in neurodegeneration, specifically their role in mediating inflammatory response to disease is unclear.

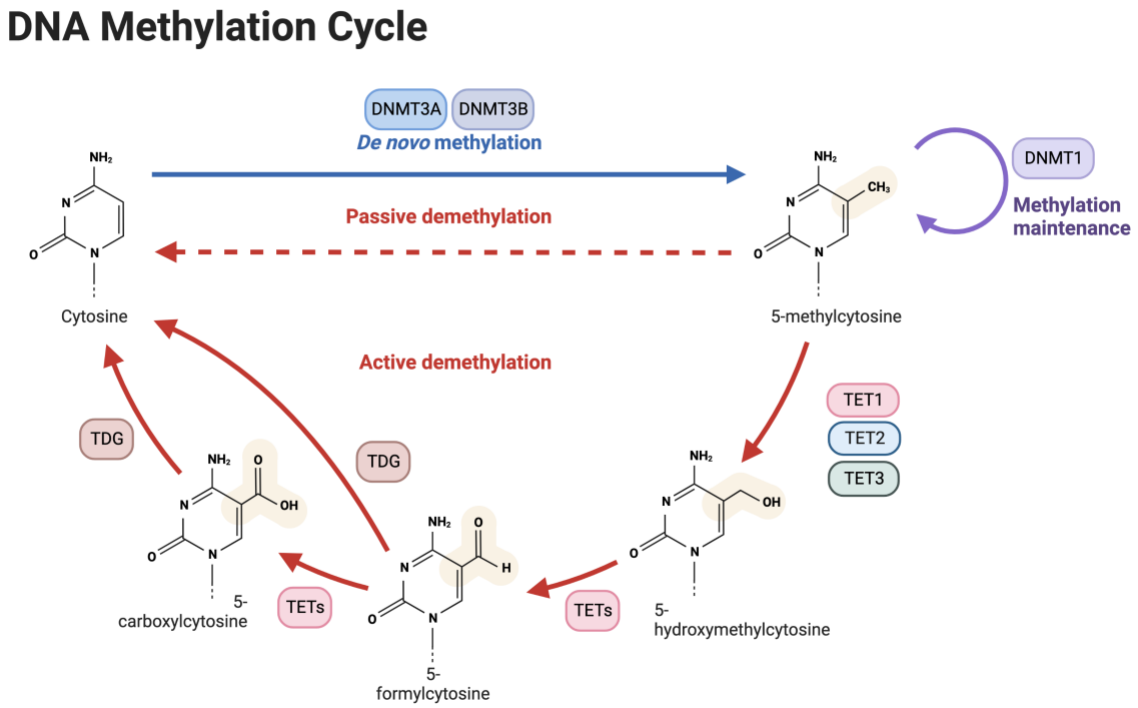


Figure 5: The DNA methylation cycle

Source: Created by Dr. Paula Desplats with Biorender.com

Previous work in Desplats lab

Previous studies in the Desplats lab have highlighted a relationship between DNA methylation and microglial inflammatory response (Figure 6A). Their research suggests that the inhibition of DNMT enzymes in a human immortalized microglial cell line (SV40) led to a decrease in levels of 5mC. This decrease in global methylation levels was shown to trigger a pro-inflammatory response, characterized by measuring the levels of cytokines TNF-α and IL6. In contrast, overexpression of DNMT1 led to an increase in 5mC levels and attenuation of the inflammatory response elicited by LPS or amyloid-Beta (Figure 6B). The findings of this study indicate that global hypermethylation may have a neuroprotective effect in the

context of Alzheimer's disease (AD). However, the mechanism by which these epigenetic changes drive the inflammatory response in microglia remains unclear.

Given the promising results of DNMT inhibition in reducing Aβ-driven response in microglial cells, my study aimed at evaluating how changes in global methylation levels contribute to driving microglial response to canonical and AD-associated inflammation, as well as explore the potential of TET inhibitors as a potential strategy against neuroinflammation and AD pathology.

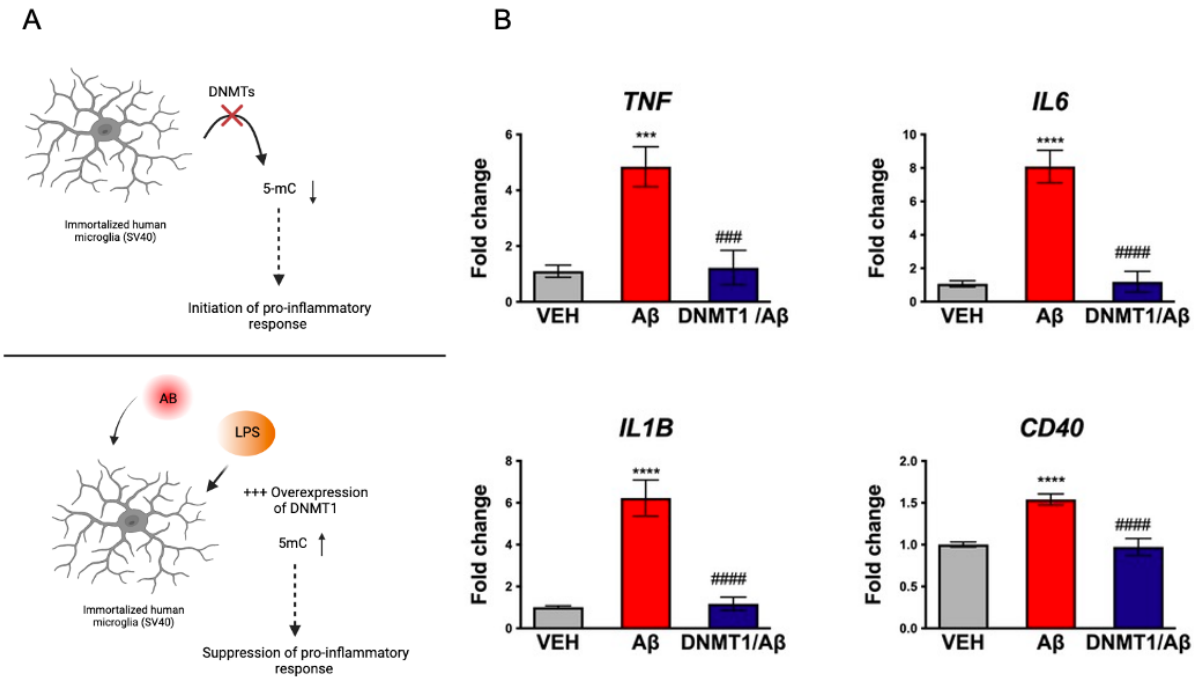


Figure 6: Previous work done in the Desplats Lab. **(A)** Graphical description of experiment **(B)** Suppression of pro-inflammatory response in SV40 cells overexpressing DNMT1 on exposure to LPS and Aβ, measured by gene expression of cytokines TNF-α, IL-6, IL1β, and CD40

Methods

Cell culture and treatment

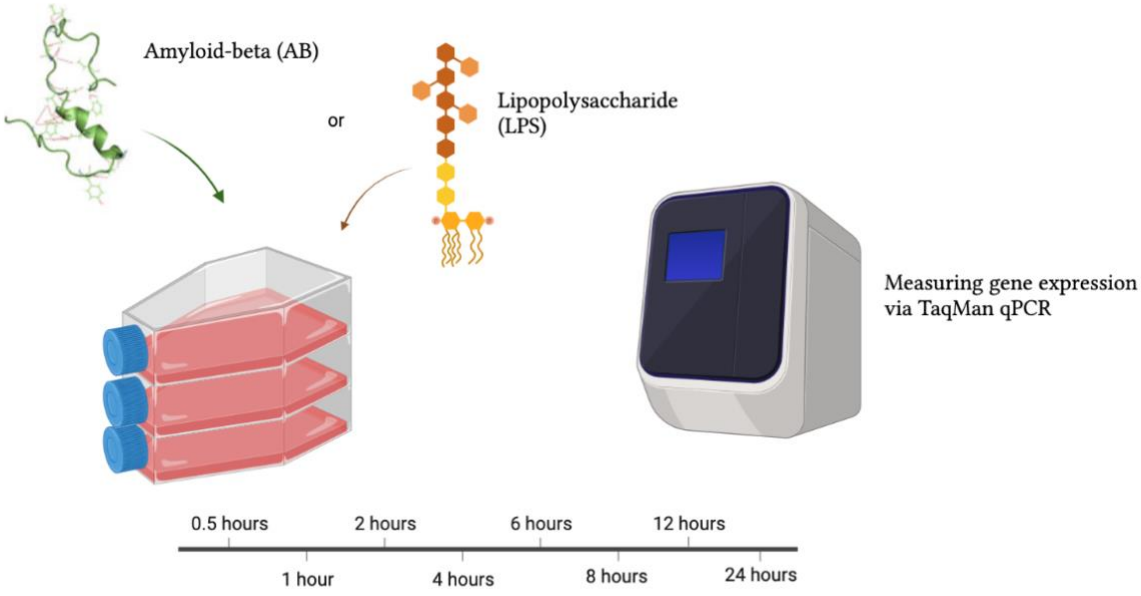
A human immortalized microglial cell line HMC3 was obtained from American Type Culture Collection (ATCC). Cells were cultured in EMEM media (ATCC) with 10% fetal bovine serum and 1% penicillin-streptomycin and incubated in a 5% CO_2 environment at 37°C. Cells were dissociated and collected using Accutase detachment reagent from Innovative Cell Technologies.

Aβ 1-42 fragments were purchased from rPeptide, dissolved in Ammonium hydroxide (NH_4OH), and cleaned using the ToxinEraser™ Endotoxin Removal Kit from GenScript. The cleaned Aβ was then diluted in culture media to a final concentration of 2.5 μM. LPS (O26:B6) was obtained from Sigma Aldrich and resuspended in PBS, and a final concentration of 100 ng/mL in culture media was used for treatment.

Amyloid-β Peptide and LPS treatments

Human microglial cells were plated at a density of 8.0 x 10^5 cells per well in a 6-well plate and grown as described above. Cultured microglial cells were treated with LPS and Aβ (diluted in media) at 100 ng/mL and 2.5 μM per well, respectively for 24 hours at 2-hour intervals (Figure 7). After a 24 h treatment, approximately 1 x10^6 cells were collected and extracted for DNA (Qiagen DNeasy Blood and Tissue Kit) and RNA (Qiagen RNeasy Plus).

Figure 7: HMC3 cells were stimulated via LPS and Aβ and collected for RNA extraction at 0.5, 1, 2, 4, 6, 8, 12, and 24 hours. Gene expression was quantified by qPCR using Taqman probes and the $2^{-\Delta\Delta Ct}$ method. Data is presented as mean values +- S.E.M. in biological triplicate.


Amyloid-beta (AB)
or
Lipopolysaccharide
(LPS)
Measuring gene expression
via TaqMan qPCR
0.5 hours
1 hour
2 hours
4 hours
6 hours
8 hours
12 hours
24 hours

Real-time PCR

The RNA was quantified with a nanodrop spectrophotometer (DeNovix) at 260nm, and 1μg was converted to cDNA using the high-capacity cDNA reverse transcription kit from Thermo Scientific. Real-time PCR reactions were then conducted using Taqman qPCR probes, Taqman Fast Advanced master mix, and the StepOne Plus Real-Time PCR system from Applied Biosystems. PCR reactions were performed in technical duplicates for all three biological triplicates. Relative quantification of gene expression was calculated using β-actin as an internal control and expressed as a fold change to the threshold cycle ($2^{-\Delta\Delta Ct}$).

Measuring 5mC and 5hmC levels using ELISA

The DNA collected from treated cells was assayed using ELISA to measure the levels of 5-mC (Active Motif Global Methylation Assay- LINE 1) and 5-hmC (Zymo Quest 5-hmC ELISA) as per the manufacturer's instructions. A standard curve was obtained, and the samples were analyzed for relative 5-hmC and 5-mC content using absorbances detected at 405 nm in a plate reader.

C35 Treatment

TET-IN-C35 (Phyllovan) from Aobious was dissolved in DMSO to a stock concentration of 10 mM. HMC3 cells were cultured in 10 cm dishes using the culture conditions described above. The cells were pre-treated with 5 μM of C35 and collected for RNA and DNA after 2 hours of LPS and Aβ 1-42 exposure. Controls were run using equal concentrations and volumes of DMSO and PBS in place of the inhibitor and LPS/Aβ respectively.

RESULTS

Characterization of the response to LPS and Aβ 1-42 fragments in HMC3 human primary immortalized microglia in culture

We first validated the human primary microglia HMC3 cells as a proper *in vitro* model for our study. To determine the appropriate time course for LPS and Aβ treatments as pro-inflammatory stimuli, we exposed HMC3 cells for 24 hours and measured the expression of two major pro-inflammatory cytokines: TNF-α and IL-6 at 2-hour intervals. TNF-α and IL-6 are pro-inflammatory cytokines that play important roles in immune regulation and tissue repair, as well as regulate the production of other neuroinflammatory mediators including reactive oxygen species and nitric oxide [57]. As shown in Figures 8A and 8B, the expression levels of both TNF and IL-6 peaked at 4 hours after LPS stimulation and returned to basal levels after around 8 hours of treatment, suggesting the resolution of inflammation.

Next, we evaluated the expression of other pro-inflammatory markers including IL1B and CD40 and DAM (Stage 1 and Stage 2) markers P2RY12, CD33, TMEM119, Cx3CR1, NOS2, LPL, CTSD, and TREM2 in HMC3 cells in response to LPS treatment in comparison to untreated cells and focusing on two points representing the peak or decay of the inflammatory response (4 and 8h, as determined above). In agreement with many research studies and literature [43, 55], exposure of HMC3 cells to LPS resulted in a significant increase of pro-inflammatory markers IL1B and CD40, DAM markers such as LPL, CTSD, and NOS2 and a significant decrease in P2ry12 and CX3CR1 (Figure 9A-9J).

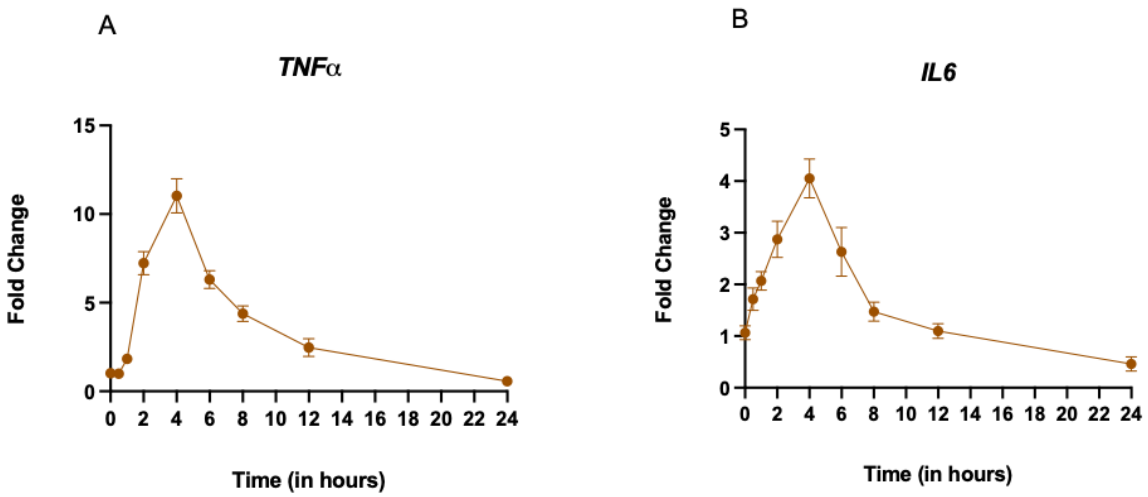


Figure 8: HMC3 cells were stimulated using 100 ng/µL LPS and collected at 0.5, 1, 2, 4, 6, 8, 12, and 24 hours. Gene expression for (A) TNF-α and (B) IL-6 was quantified by qPCR using Taqman probes and the $2^{-\Delta\Delta Ct}$ method. Data is presented as mean values +- S.E.M in biological triplicates.

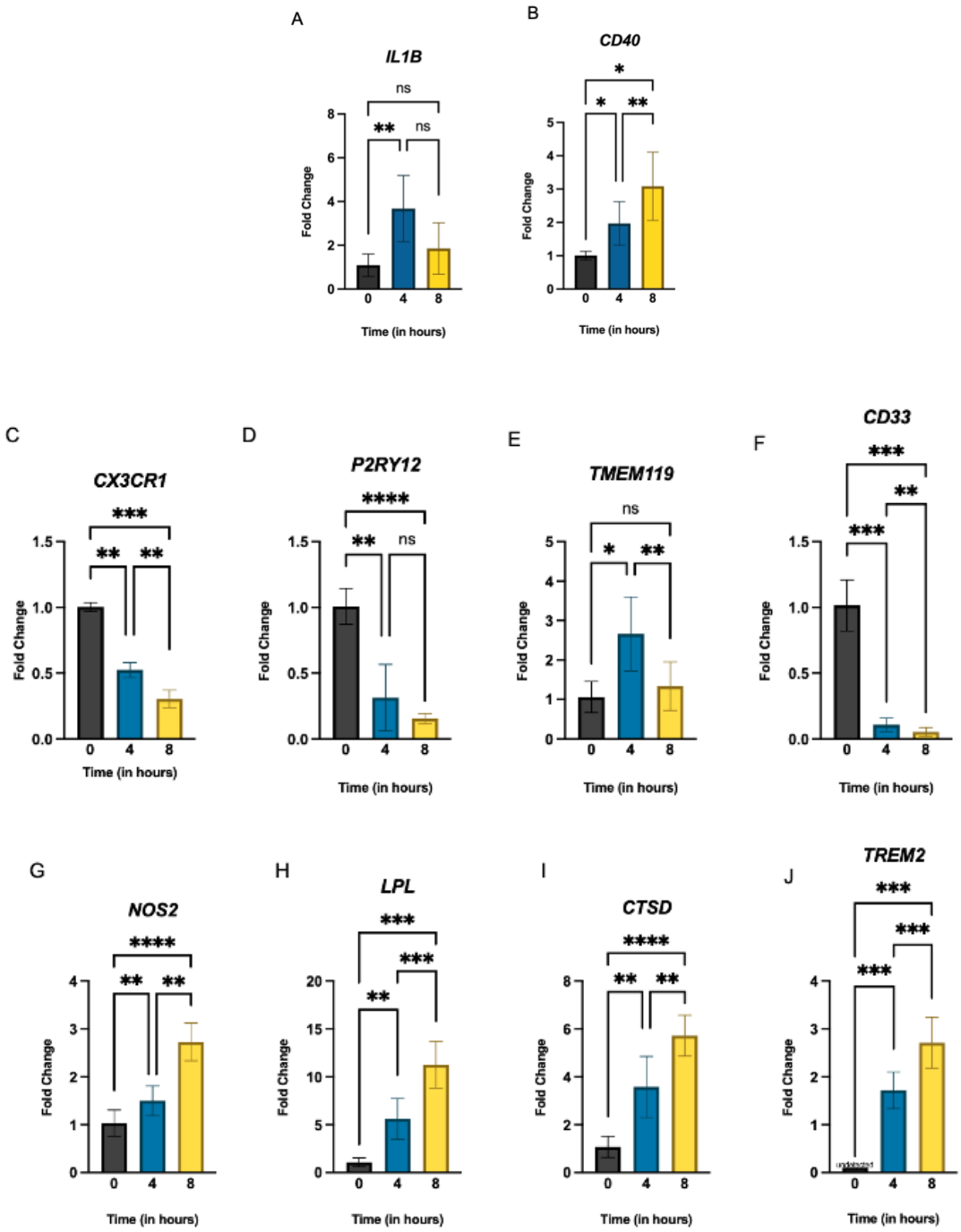


Figure 9: HMC3 cells were stimulated using 100 ng/mL LPS and collected at 0.5, 1, 2, 4, 6, 8, 12, and 24 hours. Gene expression for various inflammatory markers such as (A) IL1B and (B) CD40 and DAM markers such as (C) CX3CR1 (D) P2RY12 (E) TMEM119 (F) CD33 (G) NOS2 (H) LPL (I) CTSD and (J) TREM 2 was quantified by qPCR using Taqman probes and the $2^{-\Delta\Delta Ct}$ method. Data is presented as mean values +- S.E.M. in biological triplicates.

We next characterized this system as a potential *in vitro* model for AD, by measuring the inflammatory response to amyloid-beta. Exposure of HMC3 cells to 2.5 μM Aβ 1-42 fragments during 24 hours showed that the peak gene expression for TNF-α and IL-6 was around 2 hours, returning to basal levels by 8 hours (Figure 10A and 10B), suggesting that HMC3 cells are sensitive to Aβ exposure and the kinetics of the inflammatory response are slightly faster than to LPS. Similarly, and as shown in other microglial models, exposure of HMC3 cells to Aβ induced other molecules associated with inflammation, with a significant increase of IL1β and CD40 expression, which also reached higher levels (10-fold) in comparison to the response to LPS. In contrast, the expression levels of P2RY12, a G-coupled receptor necessary for microglial-directed motility in response to CNS injury decreased, in agreement with previous observations in postmortem brain samples from AD cases [12, 54]. Furthermore, we observed a significant increase in the expression of LPL, a Lipoprotein Lipase that is the rate-limiting enzyme in lipoprotein hydrolysis [33], and a trend for increased levels of TREM2, a gene exclusively expressed in microglia that mediates calcium signaling [56] (Figure 11A-11J). Both genes are strongly linked to a risk of AD and their upregulation followed trends reported in the literature [33, 54, 56].

These findings indicate that the human immortalized primary microglial cell line HMC3 responds to LPS and Aβ and can be used as an in-vitro model to study canonical as well as AD-induced inflammation.

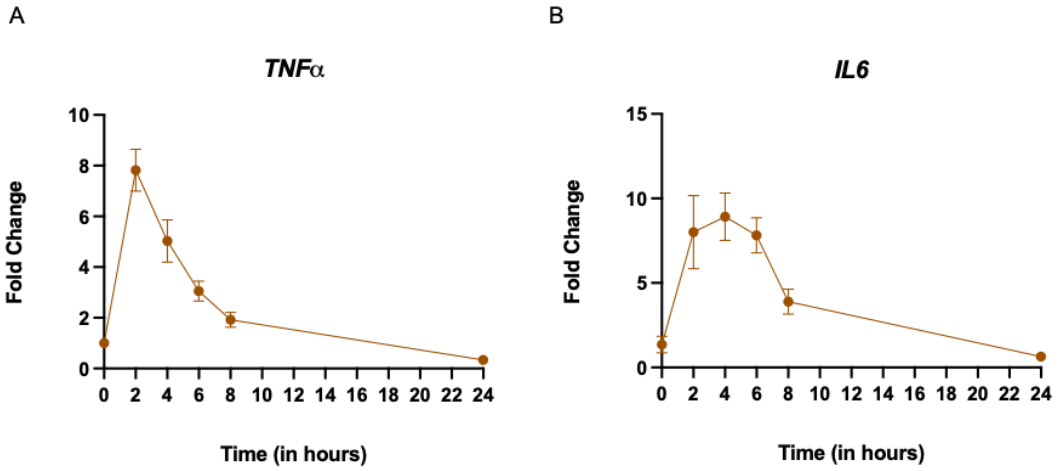


Figure 10: HMC3 cells were stimulated using 2.5 μM Aβ and collected at 0.5, 1, 2, 4, 6, 8, 12, and 24 hours. Gene expression for (A) TNF-α and (B) IL-6 was quantified by qPCR using Taqman probes and the $2^{-\Delta\Delta Ct}$ method. Data is presented as mean values +- S.E.M in biological triplicates.

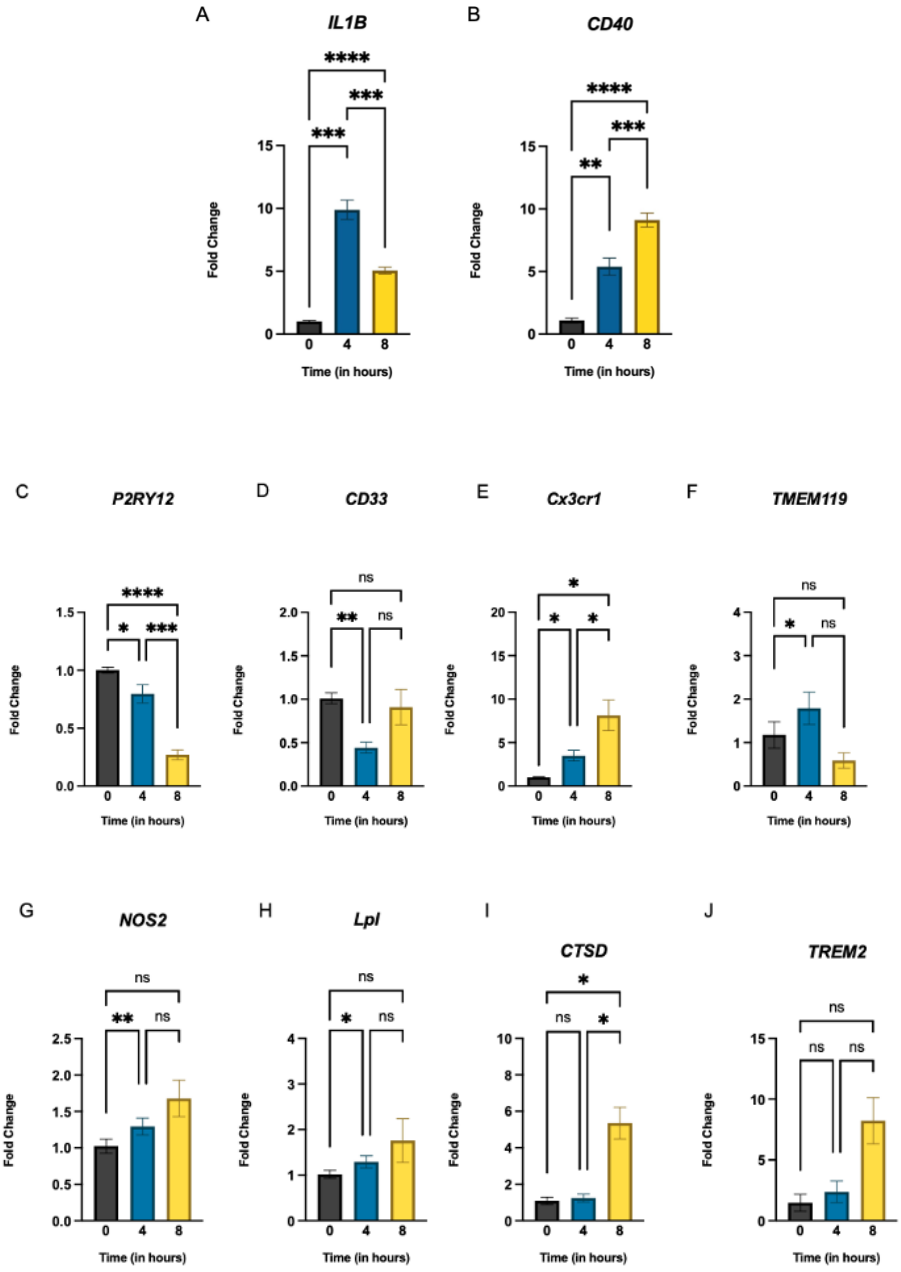


Figure 11: HMC3 cells were stimulated using 100 ng/mL LPS and collected at 0.5, 1, 2, 4, 6, 8, 12, and 24 hours. Gene expression for various inflammatory markers such as (A) IL1B and (B) CD40 and DAM markers such as (C) CX3CR1 (D) P2RY12 (E) TMEM119 (F) CD33 (G) NOS2 (H) LPL (I) CTSD and (J) TREM 2 was quantified by qPCR using Taqman probes and the $2^{-\Delta\Delta Ct}$ method. Data is presented as mean values +- S.E.M. in biological triplicates.

Microglial activation is associated with global changes in DNA methylation

We next sought to investigate the role of DNA methylation in mediating the inflammatory response in microglia. As mentioned in the previous sections, DNA methylation is a dynamic process that modulates gene expression and involves both, the addition of methyl groups by DNMTs, and the oxidation and removal of methylation by TETs. Therefore, we measured the transcriptional levels of DNMTs and TETs enzymes in HMC3 cells after exposure to LPS or Aβ using qPCR.

LPS exposure triggered an increase in the levels of DNMT3a and DNMT1 in HMC3 after 4 hours of treatment, with a trend in increased expression for DNMT3b (Figure 12A), supporting that LPS-induced inflammation elicits changes in the methylation machinery. In addition, LPS exposure induced a significant increase in the expression of TET enzymes, supporting active remodeling of DNA methylation during microglia activation (Figure 13).

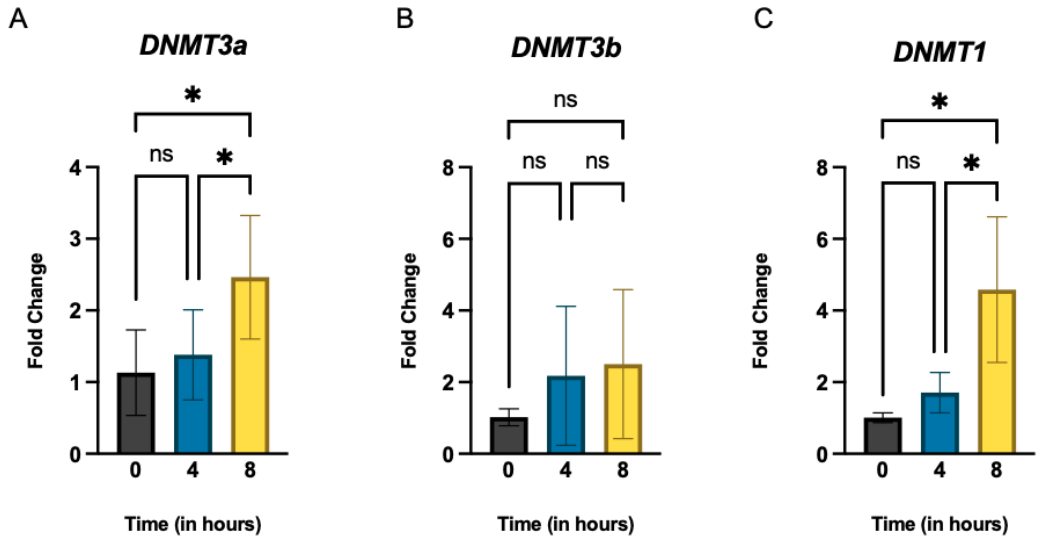


Figure 12: HMC3 cells were stimulated using 100 ng/mL LPS and collected at 0, 4 and 8 hours. Gene expression for (A) DNM3a (B) DNMT3b and (C) DNMT1 was quantified by qPCR using Taqman probes and the $2^{-\Delta\Delta Ct}$ method. Data is presented as mean values +- S.E.M. in biological triplicates.

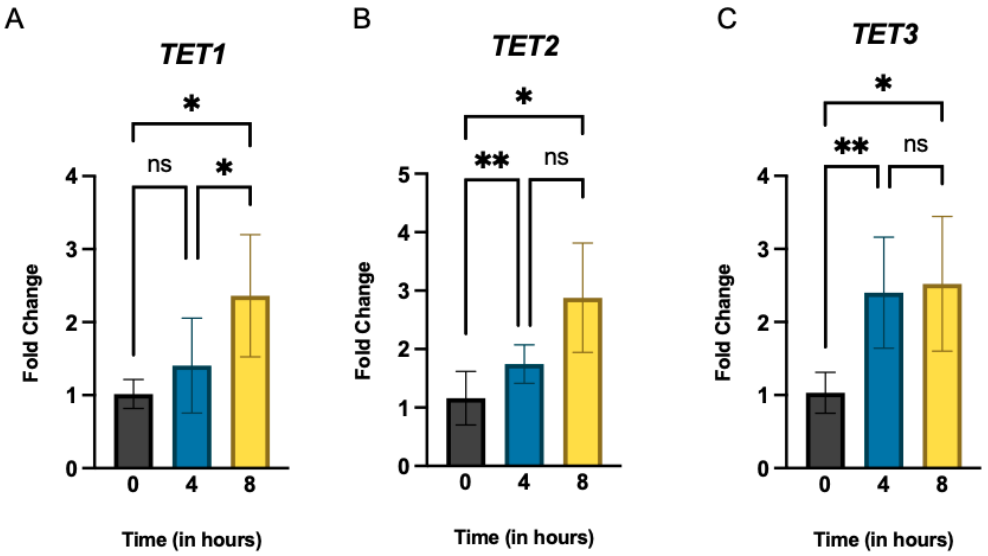


Figure 13: HMC3 cells were stimulated using 100 ng/mL LPS and collected at 0, 4 and 8 hours. Gene expression for (A) TET1 (B) TET2 and (C) TET3 was quantified by qPCR using Taqman probes and the $2^{-\Delta\Delta Ct}$ method. Data is presented as mean values +- S.E.M. in biological triplicate.

Since our primary goal was studying DNA methylation associated with AD pathology, we next investigated the expression of methylation enzymes in HMC3 cells after Aβ-treatment. Similar to what we observed in the LPS assays, exposure to Aβ induced changes in both, DNMTs and TETs. Notably, the transcription of DNMT1 was largely affected by Aβ, with increases up to 20-fold (Figure 14C). We also observed a significant increase in DNMT3b (Figure 14B), which was unaffected by LPS, suggesting that HMC3 are sensitive to AB exposure and that the mechanisms that mediate epigenetic remodeling in microglia may be dependent on the specific inflammatory stimulus.

In addition, we also observed increased expression of all TET enzymes following exposure to AB (Figure 15), suggesting that dynamic transitions between methylation and demethylation of DNA may be involved in the inflammatory response in microglia.

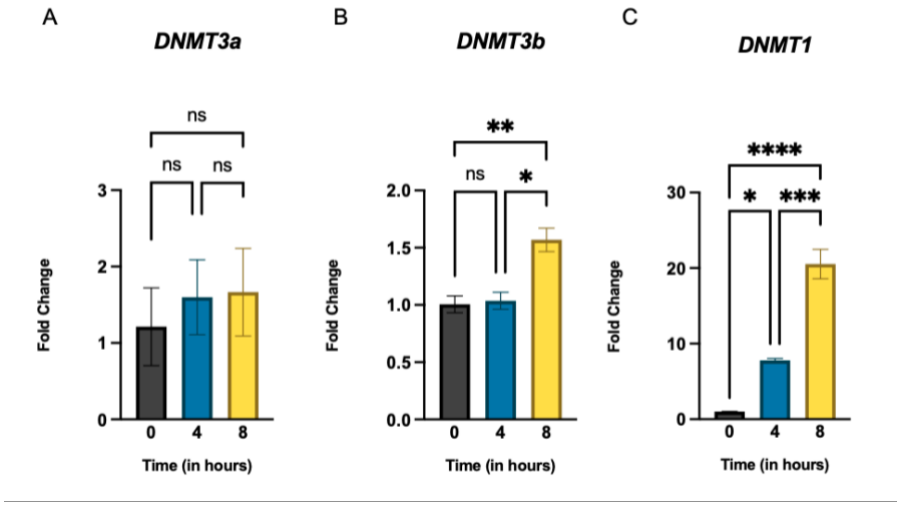


Figure 14: HMC3 cells were stimulated using 2.5 μM of Aβ 1-42 and collected at 0, 4 and 8 hours. Gene expression for (A) DNM3a (B) DNMT3b and (C) DNMT1 was quantified by qPCR using Taqman probes and the $2^{-\Delta\Delta Ct}$ method. Data is presented as mean values +- S.E.M. in biological triplicates.

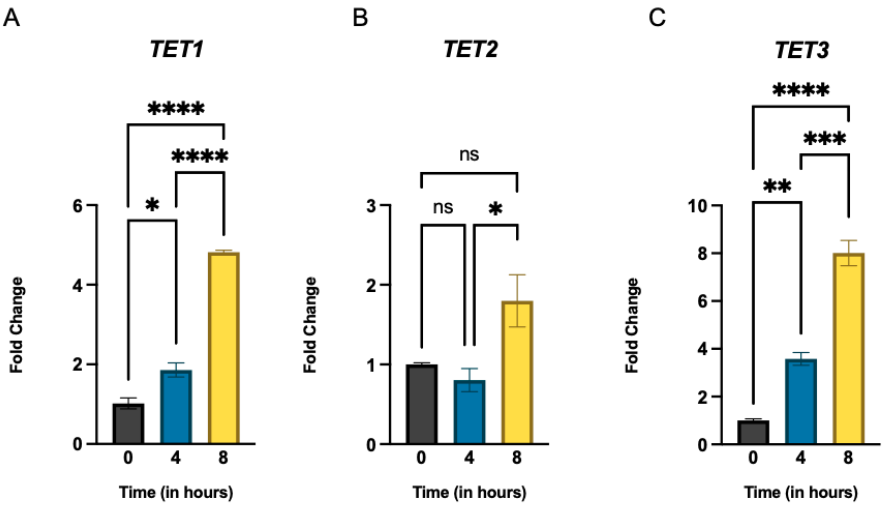


Figure 15: HMC3 cells were stimulated using 2.5 μM of Aβ 1-42 and collected at 0, 4 and 8 hours. Gene expression for (A) TET1 (B) TET2 and (C) TET3 was quantified by qPCR using Taqman probes and the $2^{-\Delta\Delta Ct}$ method. Data is presented as mean values +- S.E.M. in biological triplicates.

Evaluation of Global Methylation changes associated with Inflammation in HMC3

After observing differential expression of methylation enzymes in response to LPS or Aβ42 exposure, we next determined whether these changes impacted global levels of DNA methylation in HMC3, using ELISA assays to quantify the levels of 5mC and 5-hmC. We selected two time points, one that precedes the peak of inflammatory gene expression (2h), and one that overlaps the decay of the inflammatory expression (8h) based on our initial characterization assays. LPS induced a significant increase in 5-mC levels and an elevation in the levels of 5hmC, although this change did not reach significance (Figure 16A and 16B).

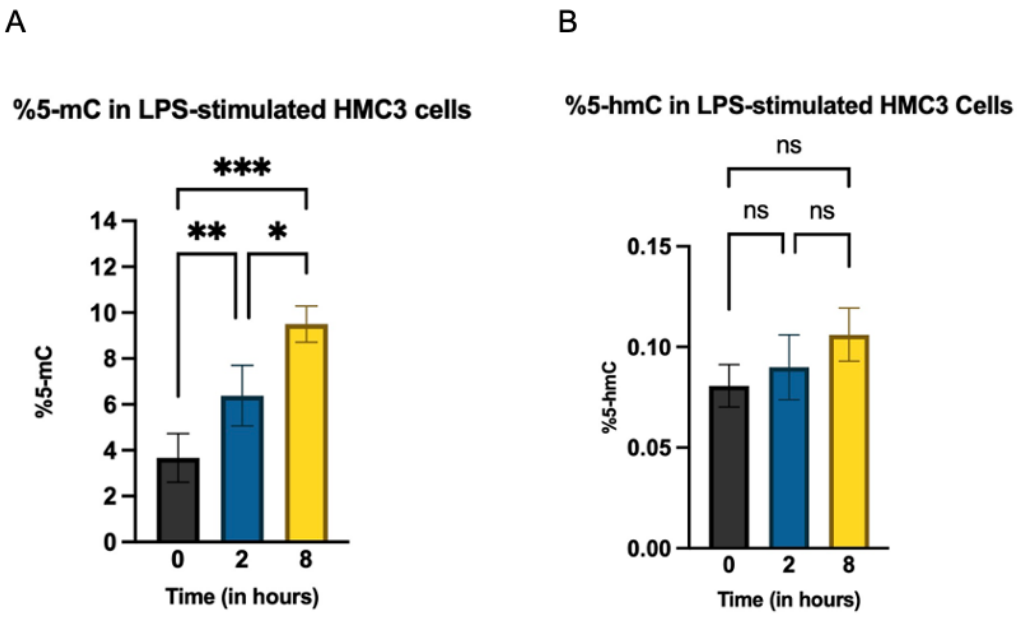


Figure 16: HMC3 cells were stimulated using 100 ng/mL LPS and collected at 0, 2, and 8 hours. (A) 5-mC and (B) 5-hmC levels were quantified using ELISA assays and their absorbance was measured using a plate reader at 405 nm.

On the other hand, Aβ42 treatment led a non-significant increase in 5-mC levels but had a more pronounced effect on the levels of 5hmC which increased significantly (Figure 17A and 17.B). This data supports a dynamic shift in DNA methylation involved in microglial activation and aligns with previous observations

from the Desplats' lab indicating that reduced methylation increases inflammation. Furthermore, it points towards inhibition of TET activity as a potential strategy to reduce microglial activation in AD.

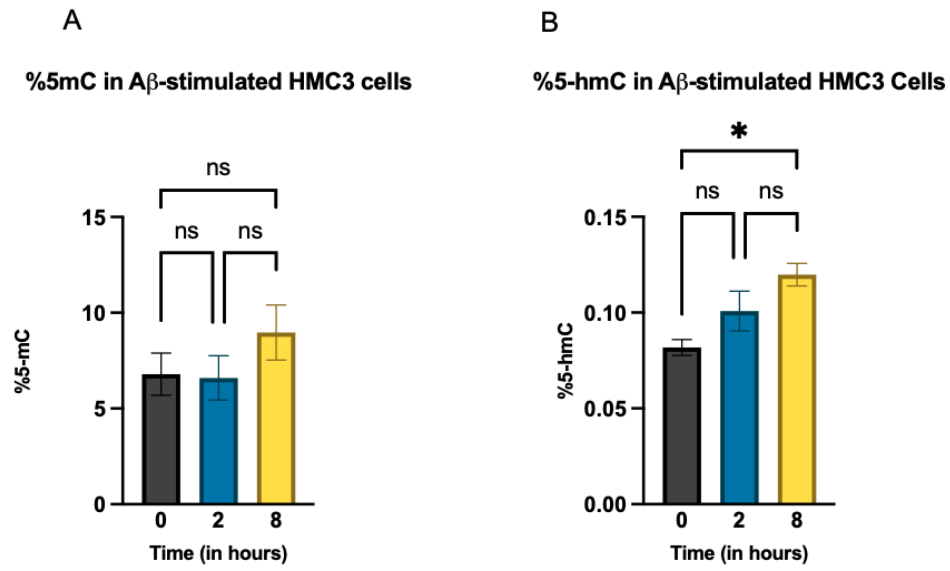


Figure 17: HMC3 cells were stimulated using 2.5 μM of Aβ 1-42 and collected at 0, 2, and 8 hours. (A) 5-mC and (B) 5-hmC levels were quantified using ELISA assays and their absorbance was measured using a plate reader at 405 nm.

Effects of TET Inhibitor C35 on Basal Methylation Levels in HMC3 Cells

While previous work of the lab in using DNMT overexpression showed promising results, our new observations of activation of TET enzymes by Aβ may cause a continuous removal of 5mc as it gets converted to 5hmC, reducing the overall levels of 5mC. Therefore, inhibition of TETs may be necessary to sustain methylation levels and to attenuate inflammation in AD. Previous studies in another neurodegenerative disorder, Parkinson's disease (PD), support this concept. A recent study [36], shows that *TET2* inactivation in a neuronal cell line results in cytosine modification changes that are reciprocal to those observed in PD neurons. Moreover, this study shows that TET2 loss in mice attenuates transcriptional immune responses to an inflammatory trigger (LPS). Lastly, the authors also observe that postmortem brain tissue from PD patients exhibits an epigenetic and transcriptional upregulation of TET2. Hence, they

suggest that decreased *Tet2* activity is neuroprotective, *in vivo*, and may be a novel therapeutic target for PD.

TET Inhibitors

Only a limited number of TET inhibitors have been developed. A recent study characterized the C35 inhibitor, a small molecule that specifically targets TET catalytic domain and reduces 5hmC load on the genome *in vitro* [51]. In this study, the authors screened the activity of C35 using a purified catalytic domain of human TET2, and also found a similar inhibitory effect of the compound on TET1 and TET3 activities after a dose course assay showed similar half-maximal inhibitory concentrations (IC_{50}) of C35 against all three TET enzymes. This suggested that C35 is a general TET inhibitor that targets all members of the TET family. Treatment of HEK293T cells stably expressing TET2 with 5 μM C35 for 3 days, showed reductions in global 5hmC levels.

Based on this data, we evaluated the changes in global methylation levels in HMC3 cells when they were treated with C35. The basal levels of 5mc and 5hmc did not change significantly after 24 hours of treatment (Figure 18A and 18.B). However, when the cells were treated for 5 days with the inhibitor, we observed a significant increase in 5mC levels, while 5hmC remained unchanged (Figure 19A and 19B). These results suggest an accumulation of 5mC as a result of blocking TET activity and further conversion into 5hmC. On the other hand, the non-significant decrease of 5hmC could be due to an arrest in the oxidative conversion of 5hmC by TETs, although further assays are needed to determine the differences observed in our studies in HMC3 with the original report in HEK293 cell overexpressing TET.

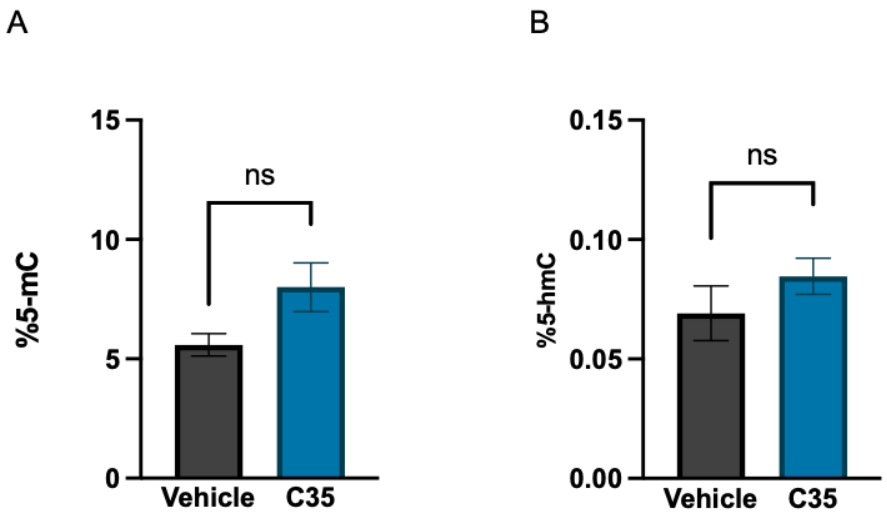


Figure 18: HMC3 cells were pre-treated with (A) DMSO (Vehicle) and (B) C35 for 24 hours and the levels of 5-mC and 5-hmC were quantified using ELISA assays and their absorbance was measured using a plate reader at 405 nm.

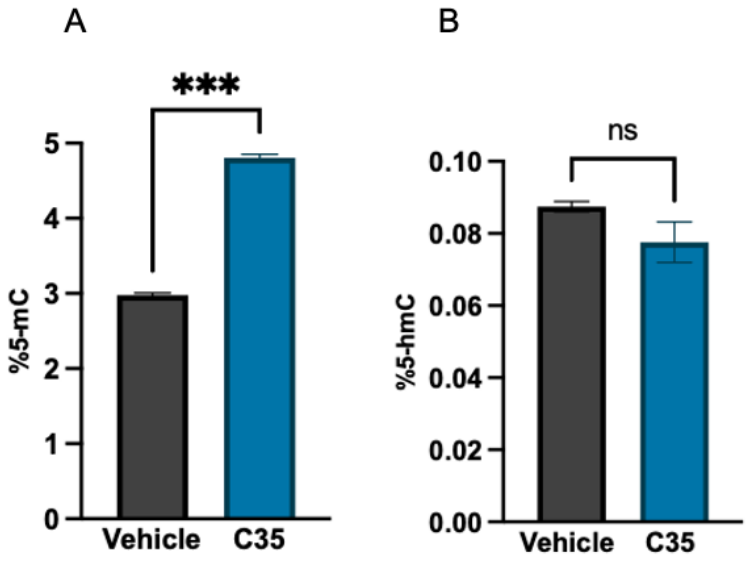


Figure 19: HMC3 cells were pre-treated with DMSO (Vehicle) and C35 for every day for 5 days and the levels of (A) 5-mC and (B) 5-hmC were quantified using ELISA assays and their absorbance was measured using a plate reader at 405 nm.

Effects of C35 treatment on methylation in HMC3 cells in response to LPS

Next, we evaluated the effects of C35 treatment on levels of 5mC and 5hmC in HMC3 cells exposed to LPS, in order to get an understanding of whether C35 can modulate the changes in methylation we observed in association with canonical inflammation. Based on our previous observations we tested the effects of C35 treatment for 24h and 5 days. HMC3 cells treated with vehicle and later exposed to LPS showed the expected increase in 5mC (Figure 20A). Treatment with C35 for 24 h before exposure to LPS (2h) did not significantly affect the increase in 5mC (Figure 20A). Furthermore, C35 treatment did not change the levels of 5hmC after LPS exposure when compared to vehicle-treated cells (Figure 20B).

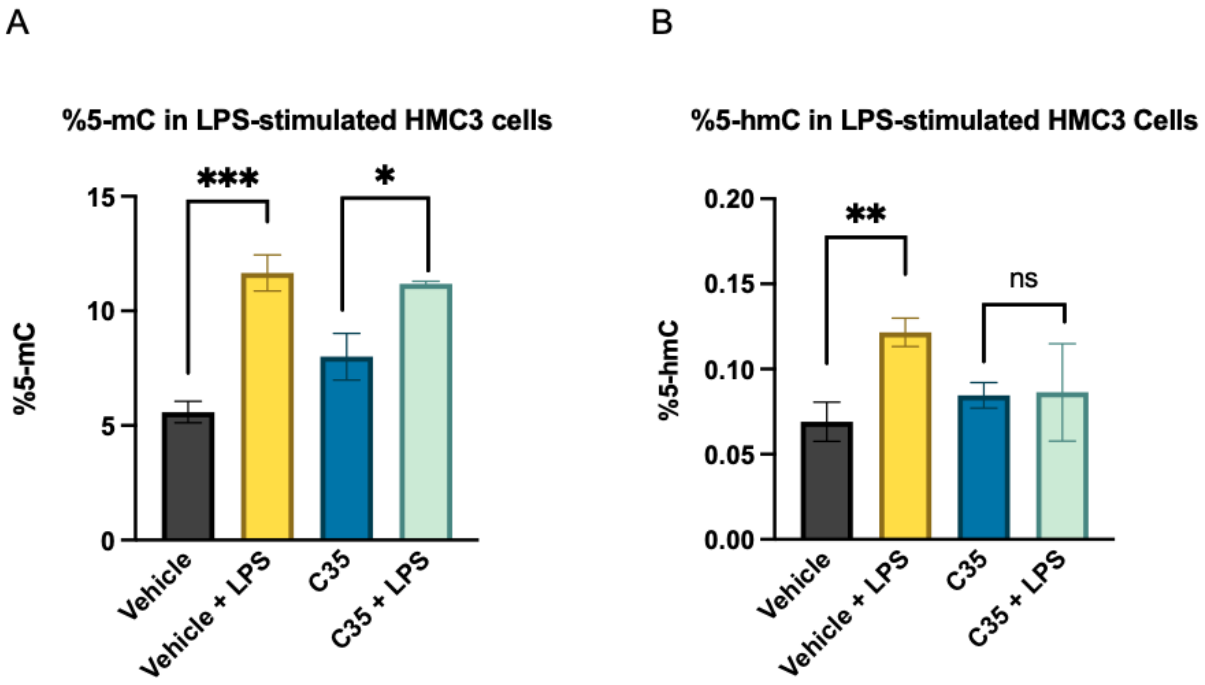


Figure 20: HMC3 cells were pre-treated with 5 μM C35 for 24 hours and stimulated with LPS. Cells were collected after 2 hours of LPS stimulation and the levels of (A) 5-mC and (B) 5-hmC were quantified for the vehicle and LPS-treated groups using ELISA assays and their absorbance was measured using a plate reader at 405 nm.

We then tested the effects of 5-day treatment with C35 on global methylation. Longer treatment with 5 μM C35 results in a larger increase in 5mC in comparison to cells treated with vehicle (Figure 21A).

Importantly, 5 days of treatment with C35 significantly decrease the levels of 5hmC in response to LPS, indicating strong inhibition of TET enzymes (Figure 21B).

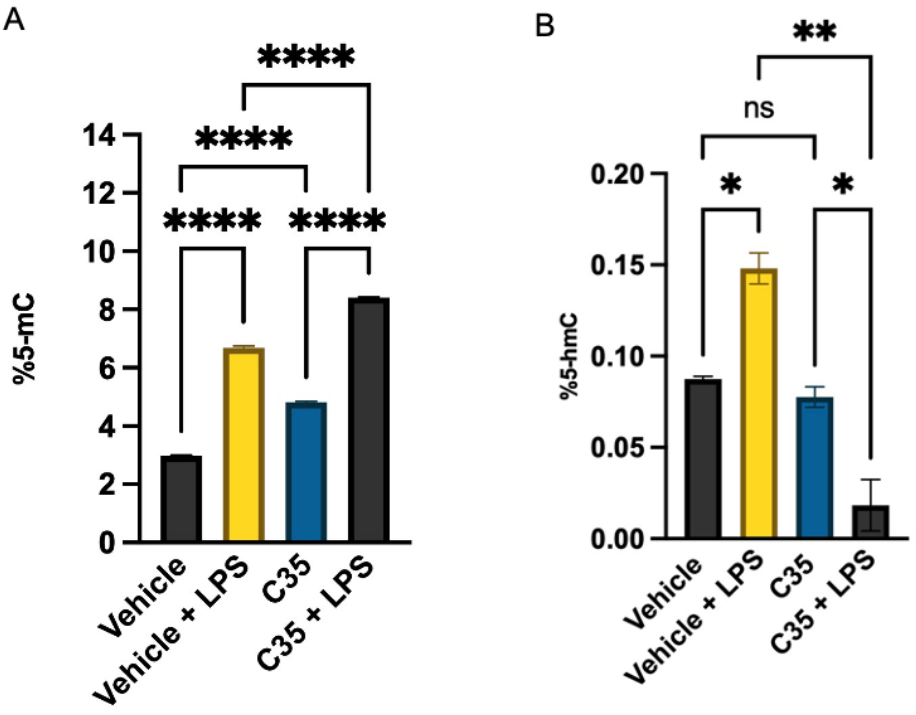


Figure 21: HMC3 cells were pre-treated with 5 μM C35 for 5 days and stimulated with LPS. Cells were collected after 2 hours of LPS stimulation and the levels of (A) 5-mC and (B) 5-hmC were quantified for the vehicle and the LPS-treated groups using ELISA assays and their absorbance was measured using a plate reader at 405 nm.

Effects of 24-hour C35 treatment on methylation in HMC3 cells in response to Aβ

Next, we evaluated the effects of 24h C35 treatment on levels of 5mC and 5hmC in HMC3 cells exposed to Aβ 1-42 (2h), to get an understanding of whether C35 can modulate the changes in methylation we observed in association with AD-associated inflammation. HMC3 cells treated with vehicle and later exposed to Aβ showed an increasing trend in 5mC levels. Treatment with C35 for 24 h before exposure to Aβ (2h) did not significantly affect the increase in 5mC (Figure 22A). Furthermore, C35 treatment did not change the levels of 5hmC after Aβ exposure when compared to vehicle-treated cells (Figure 22B). These

results suggest that a longer treatment may be necessary for efficient inhibition of TETs, as observed in LPS. It is important to note that, despite insignificant changes in global methylation in these ELISA assays, gene-specific-methylation changes may still occur.

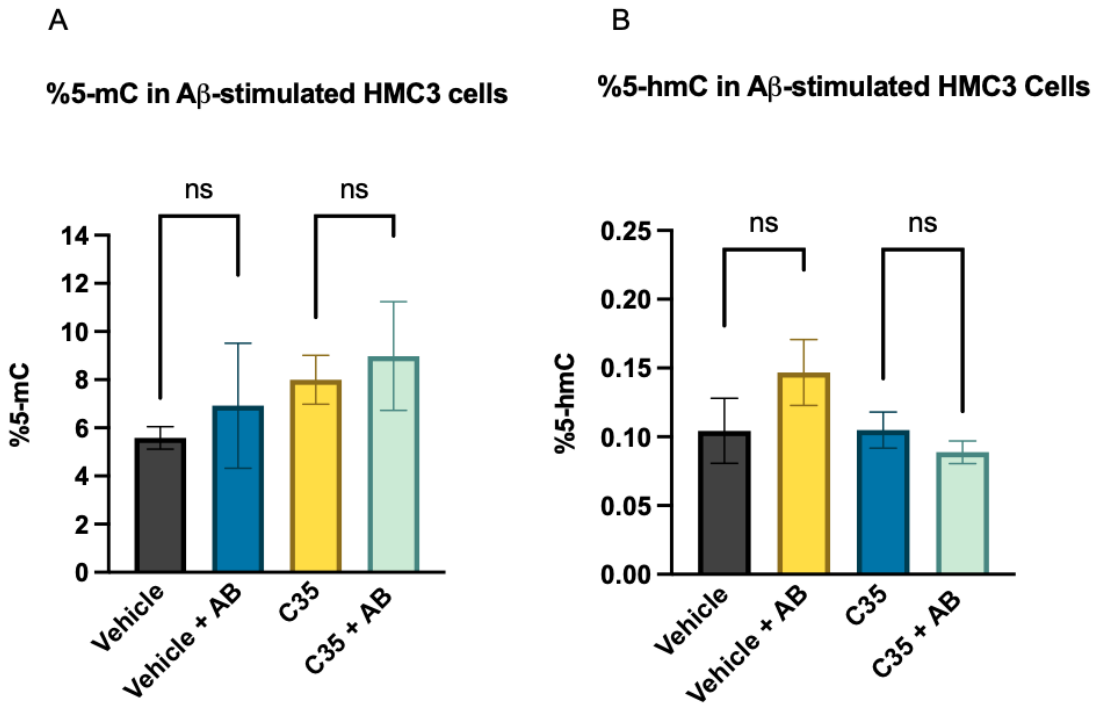


Figure 22: HMC3 cells were pre-treated with 5 μM C35 for 24 hours and stimulated with Aβ. Cells were collected after 2 hours of Aβ exposure and the levels of (A) 5-mC and (B) 5-hmC were quantified for the vehicle and the Aβ-treated groups using ELISA assays and their absorbance was measured using a plate reader at 405 nm.

Effects of C35 on the inflammatory response to LPS and Aβ in HMC3 cells

Finally, we explored whether C35-induced changes in methylation levels can attenuate the inflammatory response in microglia, both canonical and in the context of AD. LPS exposure of HMC3 cells for 2 hours saw a clear increase in TNF and IL6 expression levels (Figure 23A and 23B). The 24h C35 pre-treatment did not show a significant decrease in expression levels between the vehicle group exposed to LPS and the

pre-treated group exposed to LPS. This aligns with our observations of the lack of effect of C35 at short treatment times (24h).

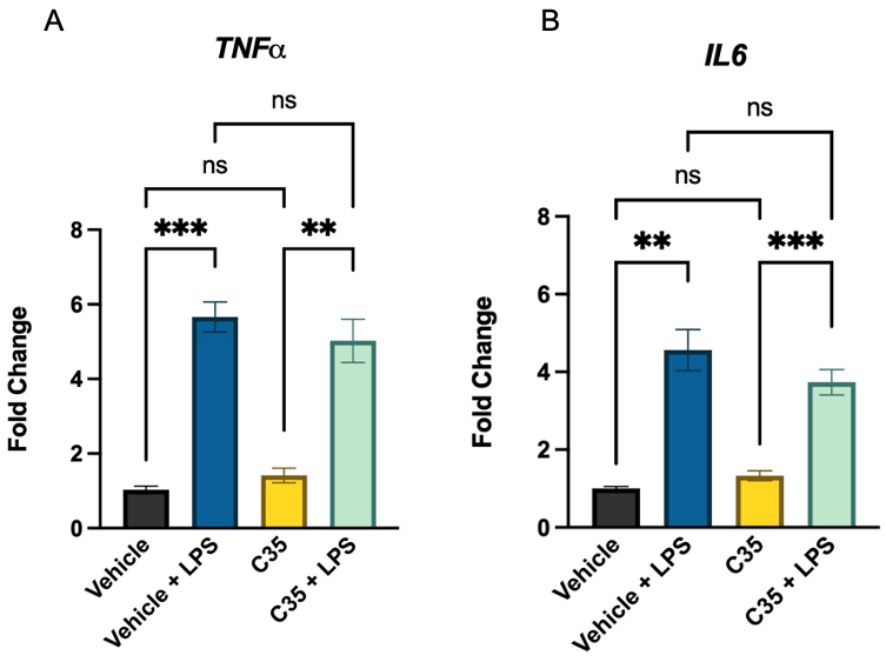


Figure 23: HMC3 cells were pre-treated with 5 μM C35 for 24 hours and stimulated with LPS. Cells were collected after 2 hours of LPS exposure and gene expression for (A) TNF-α and (B) IL-6 was quantified by qPCR using Taqman probes and the $2^{-\Delta\Delta Ct}$ method. Data is presented as mean values +- S.E.M. in biological triplicate.

On the other hand, and despite the small changes in global methylation observed, 24h treatment of HMC3 cells with 5μM C35 is sufficient to curtail the increased expression of both, TNFα and IL6 in response to Aβ, likely mediated by inhibition of TETs (Figure 24A and 24B), supporting that inhibition of TET activity may attenuate inflammation by modulation of 5hmC levels.

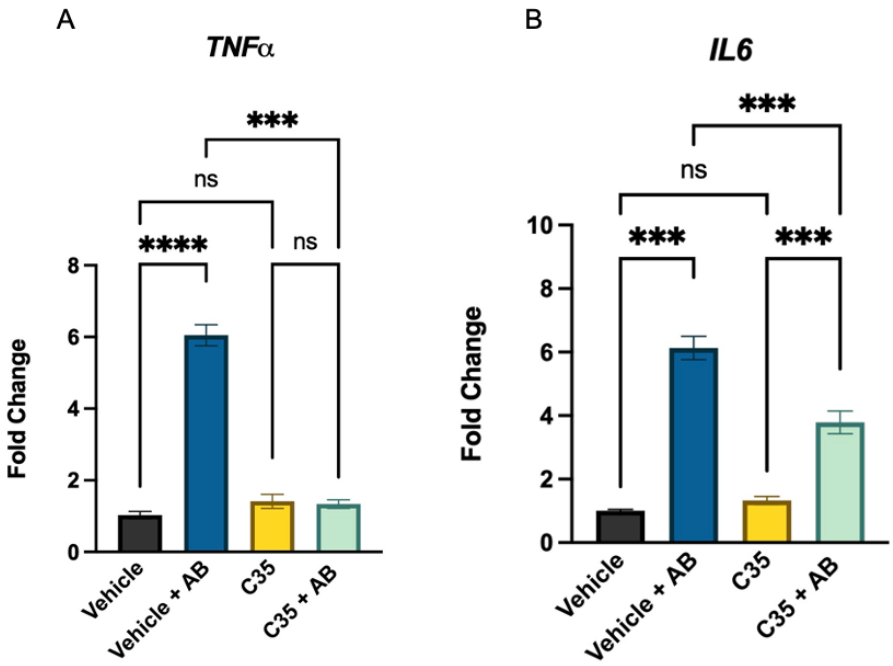


Figure 24: HMC3 cells were pre-treated with 5 μM C35 for 24 hours and stimulated with Aβ. Cells were collected after 2 hours of AB exposure and gene expression for (A) TNF-α and (B) IL-6 was quantified by qPCR using Taqman probes and the $2^{-\Delta\Delta Ct}$ method. Data is presented as mean values +- S.E.M. in biological duplicates.

Conclusion

Through this study, we show that human microglial cells (HMC3s) respond to inflammatory stimuli like LPS, but more importantly, they are highly sensitive to Amyloid-beta and can be used to model AD-associated neuroinflammation.

Further, this HMC3 inflammatory response involves changes in DNA methylation (5mC and 5hmC levels) as well as changes in the expression of DNA methylation enzymes (DNMTS and TETS). We explored the role of a TET inhibitor as a strategy to reduce neuroinflammation and observed that the TET inhibitor C35 was effective in modulating levels of 5mc and 5hmc in cell culture. More importantly, we report that C35 specifically attenuates the inflammatory response of HMC3 to Amyloid-beta. Hence, inhibition of TETs may be a potential approach to reduce neuroinflammation and pathology in AD.

Future Directions

Time and Dose Response curves for C35

As seen in cells stimulated with LPS and pre-treated with C35, the 5-day window showed a significant change in 5mc and 5-hmC levels as compared to the 24-hour treatment. Thus, additional studies including a time-course response are needed to optimize the effects of C35 in this model. In addition, different concentrations need to be assayed in the future.

Determination of DNMTs and TETs activities

Our studies only measured the levels of transcription of DNMTs and TETs in response to LPS and Aβ. Future assays measuring the activity of DNMT and TET proteins are needed to establish the role of these enzymes in inflammation.

Triculture experiments with microglia, astrocytes, and neurons

Nowadays, much research has been focused on co-culture and tri-culture models of microglia with astrocytes and neurons. The expression of certain inflammatory markers could be compared between the cell types and the way the interactions between the cell types change methylation levels.

In vivo experiments with AD-mice

So far, the work done on the C35 inhibitor has been *in vitro* in HEK293 cells, Mouse embryonic feeder cells (MEFs), and human microglia. Not much is known about the compound's ability to reach the brain, but the next step would be to test this inhibitor *in vivo* using mouse models of AD.

Sequencing for gene-specific methylation

Lastly, as mentioned before, the lack of detection of significant changes in global methylation doesn't indicate the lack of methylation in specific genes, which ultimately dictates transcription. Sequencing technologies such as RRBS (reduced representation bisulfite sequencing), whole-genome bisulfite

sequencing, or DNA methylation microarrays can be used to analyze the genome-wide methylation profiles on a single nucleotide level.

Limitations of the study

Differences in microglial behavior *in vitro* vs. *in vivo*

Cultured microglia have repeatedly been shown to behave differently than when they reside in the brain. The HMC3 cells used in this study are immortalized and present a faster replication rate than cells in vivo. Further, inside the brain, the cells receive multiple signals from the extracellular environment and other brain cell types, which cannot be modeled in a culture dish. Further, microglia have different gene expression patterns and histone modifications *in vitro* [57].

References

[1] Alonso, A. del, Zaidi, T., Novak, M., Grundke-Iqbal, I., & Iqbal, K. (2001). Hyperphosphorylation induces self-assembly of τ into tangles of paired helical filaments/straight filaments. *Proceedings of the National Academy of Sciences*, *98*(12), 6923–6928. https://doi.org/10.1073/pnas.121119298

[2] Antunes, C., Sousa, N., Pinto, L., & Marques, C. J. (2019). Tet enzymes in neurophysiology and brain function. *Neuroscience & Biobehavioral Reviews*, *102*, 337–344. https://doi.org/10.1016/j.neubiorev.2019.05.006

[3] Avila, J. (2006). Tau phosphorylation and aggregation in alzheimer's disease pathology. *FEBS Letters*, *580*(12), 2922–2927. https://doi.org/10.1016/j.febslet.2006.02.067

[4] Baker-Nigh, A., Vahedi, S., Davis, E. G., Weintraub, S., Bigio, E. H., Klein, W. L., & Geula, C. (2015). Neuronal amyloid-β accumulation within cholinergic basal forebrain in ageing and alzheimer's disease. *Brain*, *138*(6), 1722–1737. https://doi.org/10.1093/brain/awv024

[5] Breiling, A., & Lyko, F. (2015). Epigenetic regulatory functions of DNA modifications: 5-methylcytosine and beyond. *Epigenetics & Chromatin*, *8*(1). https://doi.org/10.1186/s13072-015-0016-6

[6] Butovsky, O., Jedrychowski, M. P., Moore, C. S., Cialic, R., Lanser, A. J., Gabriely, G., Koeglsperger, T., Dake, B., Wu, P. M., Doykan, C. E., Fanek, Z., Liu, L., Chen, Z., Rothstein, J. D., Ransohoff, R. M., Gygi, S. P., Antel, J. P., & Weiner, H. L. (2013). Identification of a unique TGF-β–dependent molecular and functional signature in microglia. *Nature Neuroscience*, *17*(1), 131–143. https://doi.org/10.1038/nn.3599

[7] Cheng, Y., Bernstein, A., Chen, D., & Jin, P. (2014). 5-hydroxymethylcytosine: A new player in brain disorders? *Experimental Neurology*, *268*, 3–9. https://doi.org/10.1016/j.expneurol.2014.05.008

[8] Chouliaras, L., L.A. van den Hove, D., Kenis, G., Keitel, S., R. Hof, P., van Os, J., W.M. Steinbusch, H., Schmitz, C., & P.F. Rutten, B. (2012). Age-related increase in levels of 5-hydroxymethylcytosine in mouse hippocampus is prevented by caloric restriction. *Current Alzheimer Research*, *9*(5), 536–544. https://doi.org/10.2174/156720512800618035

[9] Colom-Cadena, M., Spires-Jones, T., Zetterberg, H., Blennow, K., Caggiano, A., DeKosky, S. T., Fillit, H., Harrison, J. E., Schneider, L. S., Scheltens, P., de Haan, W., Grundman, M., van Dyck, C. H., Izzo, N. J., & Catalano, S. M. (2020). The clinical promise of biomarkers of synapse damage or loss in alzheimer's disease. *Alzheimer's Research & Therapy*, *12*(1). https://doi.org/10.1186/s13195-020-00588-4

[10] Condello, C., Yuan, P., Schain, A., & Grutzendler, J. (2015). Microglia constitute a barrier that prevents neurotoxic protofibrillar AB42 hotspots around plaques. *Nature Communications*, *6*(1). https://doi.org/10.1038/ncomms7176

[11] Coppieters, N., Dieriks, B. V., Lill, C., Faull, R. L. M., Curtis, M. A., & Dragunow, M. (2014). Global changes in DNA methylation and hydroxymethylation in alzheimer's disease human brain. *Neurobiology of Aging*, *35*(6), 1334–1344. https://doi.org/10.1016/j.neurobiolaging.2013.11.031

[12] Deczkowska, A., Keren-Shaul, H., Weiner, A., Colonna, M., Schwartz, M., & Amit, I. (2018). Disease-associated microglia: A universal immune sensor of neurodegeneration. *Cell*, *173*(5), 1073–1081. https://doi.org/10.1016/j.cell.2018.05.003

[13] D'Addario, C., & Maccarrone, M. (2016). Alcohol and epigenetic modulations. *Molecular Aspects of Alcohol and Nutrition*, 261–273. https://doi.org/10.1016/b978-0-12-800773-0.00021-5

[14] Erkkinen, M. G., Kim, M.-O., & Geschwind, M. D. (2017). Clinical neurology and epidemiology of the major neurodegenerative diseases. *Cold Spring Harbor Perspectives in Biology*, *10*(4). https://doi.org/10.1101/cshperspect.a033118

[15] Ferreira, S. T., Lourenco, M. V., Oliveira, M. M., & De Felice, F. G. (2015). Soluble amyloid-Î2 oligomers as synaptotoxins leading to cognitive impairment in Alzheimerâ€TMs disease. *Frontiers in Cellular Neuroscience*, *9*. https://doi.org/10.3389/fncel.2015.00191

[16] Grundke-Iqbal, I., Iqbal, K., Tung, Y. C., Quinlan, M., Wisniewski, H. M., & Binder, L. I. (1986). Abnormal phosphorylation of the microtubule-associated protein tau (tau) in alzheimer cytoskeletal pathology. *Proceedings of the National Academy of Sciences*, *83*(13), 4913–4917. https://doi.org/10.1073/pnas.83.13.4913

[17] Gómez Morillas, A., Besson, V. C., & Lerouet, D. (2021). Microglia and neuroinflammation: What place for p2ry12? *International Journal of Molecular Sciences*, *22*(4), 1636. https://doi.org/10.3390/ijms22041636

[18] Hampel, H., Hardy, J., Blennow, K., Chen, C., Perry, G., Kim, S. H., Villemagne, V. L., Aisen, P., Vendruscolo, M., Iwatsubo, T., Masters, C. L., Cho, M., Lannfelt, L., Cummings, J. L., & Vergallo, A. (2021). The amyloid-β pathway in alzheimer's disease. *Molecular Psychiatry*, *26*(10), 5481–5503. https://doi.org/10.1038/s41380-021-01249-0

[19] Hansen, D. V., Hanson, J. E., & Sheng, M. (2017). Microglia in alzheimer's disease. *Journal of Cell Biology*, *217*(2), 459–472. https://doi.org/10.1083/jcb.201709069

[20] Heneka, M. T., McManus, R. M., & Latz, E. (2018). Inflammasome signalling in brain function and neurodegenerative disease. *Nature Reviews Neuroscience*, *19*(10), 610–621. https://doi.org/10.1038/s41583-018-0055-7

[21] Hensley, K., Carney, J. M., Mattson, M. P., Aksenova, M., Harris, M., Wu, J. F., Floyd, R. A., & Butterfield, D. A. (1994). A model for beta-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer disease. Proc Natl Acad Sci U S A, 91(8), 3270-3274. https://doi.org/10.1073/pnas.91.8.3270

[22] Hodgson, N., Trivedi, M., Muratore, C., Li, S., & Deth, R. (2013). Soluble oligomers of amyloid-β cause changes in redox state, DNA methylation, and gene transcription by inhibiting EAAT3-mediated cysteine uptake. J Alzheimers Dis, 36(1), 197-209. https://doi.org/10.3233/jad-130101

[23] Ji, K., Akgul, G., Wollmuth, L. P., & Tsirka, S. E. (2013). Microglia actively regulate the number of functional synapses. *PLoS ONE*, *8*(2). https://doi.org/10.1371/journal.pone.0056293

[24] Jin, B., Ernst, J., Tiedemann, R. L., Xu, H., Sureshchandra, S., Kellis, M., Dalton, S., Liu, C., Choi, J.-H., & Robertson, K. D. (2012). Linking DNA methyltransferases to epigenetic marks and nucleosome structure genome-wide in human tumor cells. *Cell Reports*, *2*(5), 1411–1424. https://doi.org/10.1016/j.celrep.2012.10.017

[25] Jonsson, T., Atwal, J. K., Steinberg, S., Snaedal, J., Jonsson, P. V., Bjornsson, S., Stefansson, H., Sulem, P., Gudbjartsson, D., Maloney, J., Hoyte, K., Gustafson, A., Liu, Y., Lu, Y., Bhangale, T., Graham, R. R., Huttenlocher, J., Bjornsdottir, G., Andreassen, O. A., . . . Stefansson, K. (2012). A mutation in APP protects against Alzheimer's disease and age-related cognitive decline. Nature, 488(7409), 96-99. https://doi.org/10.1038/nature11283

[26] Kumar A, Sidhu J, Goyal A, Tsao J. Alzheimer Disease. [Updated 2022 Jun 5]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK499922/

[27] Keren-Shaul, H., Spinrad, A., Weiner, A., Matcovitch-Natan, O., Dvir-Szternfeld, R., Ulland, T. K., David, E., Baruch, K., Lara-Astaiso, D., Toth, B., Itzkovitz, S., Colonna, M., Schwartz, M., & Amit, I. (2017). A unique microglia type associated with restricting development of alzheimer's disease. *Cell*, *169*(7). https://doi.org/10.1016/j.cell.2017.05.018

[28] Kohli, R. M., & Zhang, Y. (2013). Tet enzymes, TDG and the dynamics of DNA demethylation. *Nature*, *502*(7472), 472–479. https://doi.org/10.1038/nature12750

[29] Lee, C. Y., & Landreth, G. E. (2010). The role of microglia in amyloid clearance from the AD brain. *Journal of Neural Transmission*, *117*(8), 949–960. https://doi.org/10.1007/s00702-010-0433-4

[30] Lippens, G., Sillen, A., Landrieu, I., Amniai, L., Sibille, N., Barbier, P., Leroy, A., Hanoulle, X., & Wieruszeski, J.-M. (2007). Tau aggregation in alzheimer's disease. *Prion*, *1*(1), 21–25. https://doi.org/10.4161/pri.1.1.4055

[31] Liu, L., Drouet, V., Wu, J. W., Witter, M. P., Small, S. A., Clelland, C., & Duff, K. (2012). Trans-synaptic spread of tau pathology in vivo. *PLoS ONE*, *7*(2). https://doi.org/10.1371/journal.pone.0031302

[32] Lively, S., & Schlichter, L. C. (2018). Microglia responses to pro-inflammatory stimuli (LPS, IFNΓ+TNFα) and reprogramming by resolving cytokines (IL-4, IL-10). *Frontiers in Cellular Neuroscience*, *12*. https://doi.org/10.3389/fncel.2018.00215

[33] Loving, B. A., Tang, M., Neal, M. C., Gorkhali, S., Murphy, R., Eckel, R. H., & Bruce, K. D. (2021). Lipoprotein lipase regulates microglial lipid droplet accumulation. *Cells*, *10*(2), 198. https://doi.org/10.3390/cells10020198

[34] Lue, L. F., Kuo, Y. M., Roher, A. E., Brachova, L., Shen, Y., Sue, L., Beach, T., Kurth, J. H., Rydel, R. E., & Rogers, J. (1999). Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. Am J Pathol, 155(3), 853-862. https://doi.org/10.1016/s0002-9440(10)65184-x

[35] Mandelkow, E. (1998). Tau in alzheimer's disease. *Trends in Cell Biology*, *8*(11), 425–427. https://doi.org/10.1016/s0962-8924(98)01368-3

[36] Marshall, L. L., Killinger, B. A., Ensink, E., Li, P., Li, K. X., Cui, W., Lubben, N., Weiland, M., Wang, X., Gordevicius, J., Coetzee, G. A., Ma, J., Jovinge, S., & Labrie, V. (2020). Epigenomic analysis of parkinson's disease neurons identifies TET2 loss as neuroprotective. *Nature Neuroscience*, *23*(10), 1203–1214. https://doi.org/10.1038/s41593-020-0690-y

[37] Mattson, M. P., Carney, J. W., & Butterfield, D. A. (1995). A tombstone in alzheimer's? *Nature*, *373*(6514), 481–481. https://doi.org/10.1038/373481a0

[38] Mayeux, R., & Stern, Y. (2012). Epidemiology of alzheimer disease. *Cold Spring Harbor Perspectives in Medicine*, *2*(8). https://doi.org/10.1101/cshperspect.a006239

[39] Medeiros, R., Baglietto-Vargas, D., & LaFerla, F. M. (2011). The role of tau in alzheimer's disease and related disorders. *CNS Neuroscience Therapeutics*, *17*(5), 514–524. https://doi.org/10.1111/j.1755-5949.2010.00177.x

[40] Moore, L. D., Le, T., & Fan, G. (2012). DNA methylation and its basic function. *Neuropsychopharmacology*, *38*(1), 23–38. https://doi.org/10.1038/npp.2012.112

[41] Niikura, T., Hashimoto, Y., Tajima, H., & Nishimoto, I. (2002). Death and survival of neuronal cells exposed to alzheimer's insults. *Journal of Neuroscience Research*, *70*(3), 380–391. https://doi.org/10.1002/jnr.10354

[42] Pike, C. J., Walencewicz-Wasserman, A. J., Kosmoski, J., Cribbs, D. H., Glabe, C. G., & Cotman, C. W. (2002). Structure-activity analyses of β-amyloid peptides: Contributions of

the B25-35 region to aggregation and neurotoxicity. *Journal of Neurochemistry*, *64*(1), 253–265. https://doi.org/10.1046/j.1471-4159.1995.64010253.x

[43] Qin, H., Wilson, C. A., Lee, S. J., Zhao, X., & Benveniste, E. N. (2005). LPS induces CD40 gene expression through the activation of NF-KB and STAT-1α in macrophages and microglia. *Blood*, *106*(9), 3114–3122. https://doi.org/10.1182/blood-2005-02-0759

[44] Rasmussen, K. D., & Helin, K. (2016). Role of TET enzymes in DNA methylation, development, and cancer. *Genes & Development*, *30*(7), 733–750. https://doi.org/10.1101/gad.276568.115

[45] Sakono, M., & Zako, T. (2010). Amyloid oligomers: Formation and toxicity of AB oligomers. *FEBS Journal*, *277*(6), 1348–1358. https://doi.org/10.1111/j.1742-4658.2010.07568.x

[46] Schafer, D. P., Lehrman, E. K., Kautzman, A. G., Koyama, R., Mardinly, A. R., Yamasaki, R., Ransohoff, R. M., Greenberg, M. E., Barres, B. A., & Stevens, B. (2012). Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. *Neuron*, *74*(4), 691–705. https://doi.org/10.1016/j.neuron.2012.03.026

[47] Sengupta, U., Nilson, A. N., & Kayed, R. (2016). The role of amyloid-β oligomers in toxicity, propagation, and immunotherapy. *EBioMedicine*, *6*, 42–49. https://doi.org/10.1016/j.ebiom.2016.03.035

[48] Serrano-Pozo, A., Frosch, M. P., Masliah, E., & Hyman, B. T. (2011). Neuropathological alterations in alzheimer disease. *Cold Spring Harbor Perspectives in Medicine*, *1*(1). https://doi.org/10.1101/cshperspect.a006189

[49] Sharma, V. K., Mehta, V., & Singh, T. G. (2020). Alzheimer's disorder: Epigenetic connection and associated risk factors. *Current Neuropharmacology*, *18*(8), 740–753. https://doi.org/10.2174/1570159x18666200128125641

[50] Shi, Q., Chowdhury, S., Ma, R., Le, K. X., Hong, S., Caldarone, B. J., Stevens, B., & Lemere, C. A. (2017). Complement C3 deficiency protects against neurodegeneration in aged plaque-rich app/PS1 mice. *Science Translational Medicine*, *9*(392). https://doi.org/10.1126/scitranslmed.aaf6295

[51] Singh, A. K., Zhao, B., Liu, X., Wang, X., Li, H., Qin, H., Wu, X., Ma, Y., Horne, D., & Yu, X. (2020). Selective targeting of Tet catalytic domain promotes somatic cell reprogramming. *Proceedings of the National Academy of Sciences*, *117*(7), 3621–3626. https://doi.org/10.1073/pnas.1910702117

[52] Szulwach, K. E., Li, X., Li, Y., Song, C.-X., Wu, H., Dai, Q., Irier, H., Upadhyay, A. K., Gearing, M., Levey, A. I., Vasanthakumar, A., Godley, L. A., Chang, Q., Cheng, X., He, C., & Jin, P. (2011). 5-HMC–mediated epigenetic dynamics during postnatal

neurodevelopment and aging. *Nature Neuroscience*, *14*(12), 1607–1616. https://doi.org/10.1038/nn.2959

[53] Takahashi, R. H., Nagao, T., & Gouras, G. K. (2017). Plaque Formation and the intraneuronal accumulation of β-amyloid in alzheimer's disease. *Pathology International*, *67*(4), 185–193. https://doi.org/10.1111/pin.12520

[54] Tozaki-Saitoh, H., Tsuda, M., Miyata, H., Ueda, K., Kohsaka, S., & Inoue, K. (2008). P2Y12receptors in spinal microglia are required for neuropathic pain after peripheral nerve injury. *The Journal of Neuroscience*, *28*(19), 4949–4956. https://doi.org/10.1523/jneurosci.0323-08.2008

[55] Tsai, Y. J., Gao, P., Williams, M. A., Grigoryev, D., Stockton, M., Watkins, T., Cheadle, C., Georas, S., Liu, M. C., Breslin, L., & Barnes, K. C. (2007). The interleukin 1β gene (IL1B) is upregulated in CD14+ monocytes but not alveolar macrophages after lipopolysaccharide (LPS) stimulation by genomic expression profiling. *Journal of Allergy and Clinical Immunology*, *119*(1), 175–176. https://doi.org/10.1016/j.jaci.2006.12.050

[56] Ulland, T. K., & Colonna, M. (2018). Trem2 — a key player in microglial biology and alzheimer disease. *Nature Reviews Neurology*, *14*(11), 667–675. https://doi.org/10.1038/s41582-018-0072-1

[57] Walsh, D. M., & Selkoe, D. J. (2004). Deciphering the molecular basis of memory failure in alzheimer's disease. *Neuron*, *44*(1), 181–193. https://doi.org/10.1016/j.neuron.2004.09.010

[58] Wang, Z., Miu, K.-K., Chan, S.-W., Ou, F., Wai-Nok Law, P., & Chan, W.-Y. (2022). Genome-wide 5-hydroxymethylcytosine (5HMC) reassigned in Pten-depleted mescs along neural differentiation. *Frontiers in Cell and Developmental Biology*, *10*. https://doi.org/10.3389/fcell.2022.956604

[59] Weller, J., & Budson, A. (2018). Current understanding of alzheimer's disease diagnosis and treatment. *F1000Research*, *7*, 1161. https://doi.org/10.12688/f1000research.14506.1

[60] Wenger, K., Viode, A., Schlaffner, C. N., van Zalm, P., Cheng, L., Dellovade, T., Langlois, X., Bannon, A., Chang, R., Connors, T. R., Oakley, D., Renard, B., Rappsilber, J., Hyman, B., Steen, H., & Steen, J. A. (2023). Common mouse models of Tauopathy reflect early but not late human disease. *Molecular Neurodegeneration*, *18*(1). https://doi.org/10.1186/s13024-023-00601-y